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Discovery of the Orally Effective Thyrotropin-Releasing Hormone Mimetic: 1-{*N*-[(4*S*,5*S*)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2*R*)-2-methylpyrrolidine Trihydrate (Rovatirelin Hydrate)

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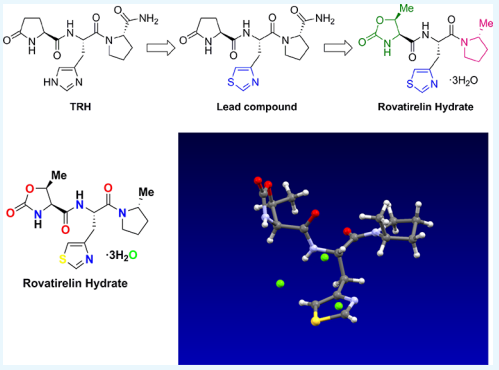
Supporting Information

ABSTRACT: We have explored orally effective thyrotropin-releasing hormone (TRH) mimetics, showing oral bioavailability and brain penetration by structure−activity relationship (SAR) study on the basis of in vivo antagonistic activity on reserpine-induced hypothermia in mice. By primary screening of the synthesized TRH mimetics, we found a novel TRH mimetic: L-pyroglutamyl-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide with a high central nervous system effect compared with TRH as a lead compound. Further SAR optimization studies of this lead compound led to discovery of a novel orally effective TRH mimetic: 1-{*N*-[(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2*R*)-2-methylpyrrolidine trihydrate (rovatirelin hydrate), which was selected as a candidate for clinical trials.



INTRODUCTION

Thyrotropin-releasing hormone (TRH), first isolated from pig or sheep, is a hypothalamic hormone that is synthesized in various areas of the brain. TRH, composed of L-pyroglutamate, L-histidine and L-prolinamide (L-pGlu−L-His−L-Pro-NH_2) (Figure 1),[1,2] is the smallest molecule among the known peptide hormones. TRH is distributed in high concentrations in the brain and accelerates the synthesis and secretion of thyrotropin-stimulating hormone (TSH)[3] and prolactin from the anterior pituitary. TRH also displays physiological activities on the central nervous system (CNS), acting as a neurotransmitter or a neuromodulator,[4−6] and has been used for the treatment of CNS disorders. Intact TRH (protirelin tartrate)[7] has been clinically used for symptomatic therapy of spinocerebellar degeneration (SCD) in Japan.

It has been reported that two subtypes of TRH receptor (TRH receptor type 1: TRH-R1[8] and TRH receptor type 2: TRH-R2[9,10]) exist and TRH-R1 is related to endocrine effects and TRH-R2 to CNS effects.[11] On the other hand, it has been reported that a single type of TRH receptor is expressed in humans and that the human TRH receptor is similar to TRH-R1.[12,13] To separate the CNS and the endocrine effects, a large number of TRH analogues such as taltirelin (TA-0910),[14] orotirelin (CG-3509),[15] montirelin (CG-3707),[16] DN-1417,[17] azetirelin (YM-14673),[18] JTP-2942,[19] posatirelin (RGH-2202),[20] MK-771,[21] and RX77368[22] were synthesized and developed before 2000. Because of the short half-life times in serum, low lipophilicity, and oral absorption, most TRH mimetics have been developed as intravenous injections and not for oral administration. Against such a background, we have been carrying out structure−activity relationship (SAR) studies with TRH to find orally effective TRH mimetics since the 1990s. Among them, only taltirelin hydrate (CEREDIST), which is an agonist at the human TRH-R has been launched in Japan as an orally effective agent for treatment of SCD (Figure 1).[14,23,24] After 2000, new types of TRH analogues such as NP-647, which binds to TRH-R subtype selectively and has only CNS effects[25] or prodrugs for brain targeting,[26] were found and evaluated.

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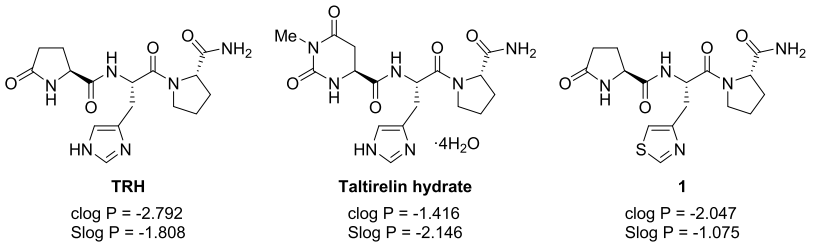


Figure 1. Chemical structure of TRH (left), taltirelin hydrate (middle) and lead compound **1** (right) and their lipophilicities.

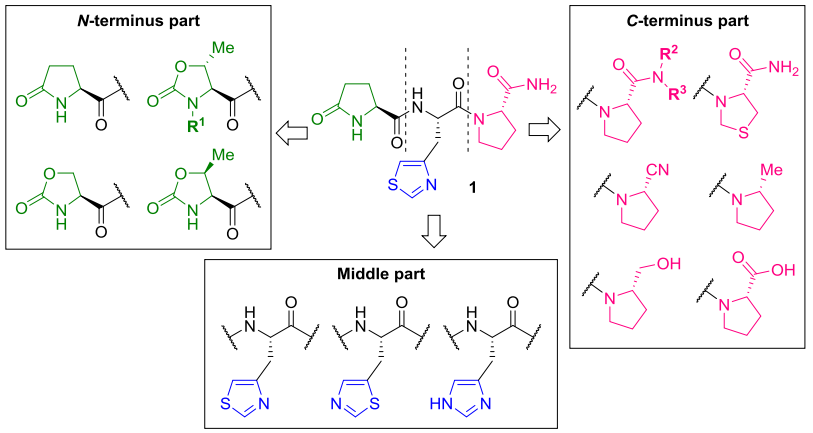
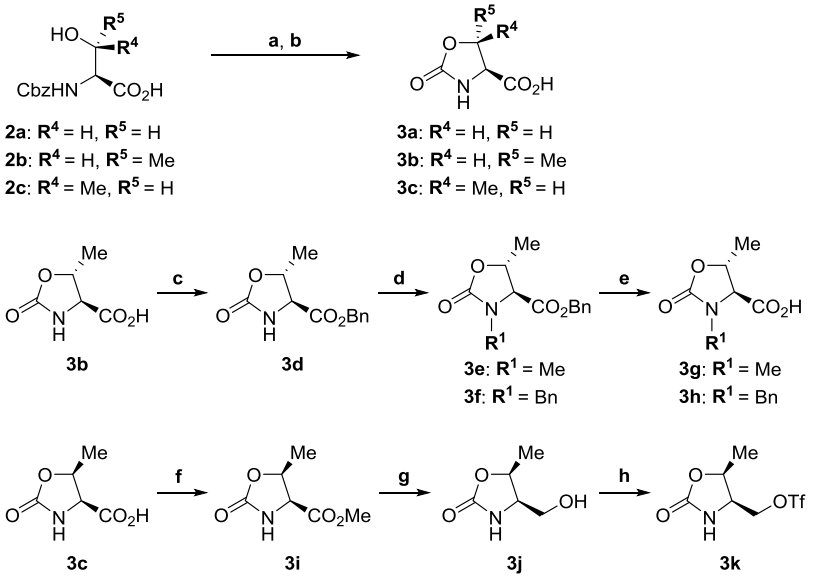


Figure 2. Outline of the SAR study from lead compound **1**.

Scheme 1. Synthesis of N-Terminus Fragments 3a−k[a]



[a]Reagents and conditions: (a) aq. NaOH, MeOH, rt; (b) aq. HCl, 49−73%; (c) BnOH, DMAP, DCC, DMF, rt, 91%; (d) MeI or BnBr, NaH, DMF, 0 °C, 86−88%; (e) H_2, Pd−C, MeOH, rt or aq. LiOH, THF-DME, rt, 80−98%; (f) $SOCl_2$, MeOH, 0 °C to rt, quant; (g) $NaBH_4$, EtOH, 0 °C, 77%; and (h) Tf_2O, pyridine, CH_2Cl_2, −35 °C, 45%.

In a primary screening study, we found a novel TRH mimetic: L-pyroglutamyl-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide **1**[27] (Figure 1), which was used as a lead compound. It has an unnatural amino acid 3-(thiazol-4-yl)-L-alanine moiety in the middle part of TRH instead of L-histidine. The most important feature of TRH mimetic **1** is its better lipophilicity than TRH, and **1** was found to have high in vivo CNS effect (antagonistic effect on reserpine-induced hypothermia) compared with TRH (see Table 1).

In this paper, we report a SAR study of lead compound **1** on the basis of its physicochemical properties, in vivo antagonistic activity on reserpine-induced hypothermia in mice, TRH-R binding affinity, and pharmacokinetic properties to find orally effective TRH mimetics (Figure 2). We finally selected a TRH mimetic 1-{*N*-[(4*S*,5*S*)-(5-methyl-2-oxooxazolidin-4-yl)-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2*R*)-2-methylpyrrolidine trihydrate (rovatirelin hydrate) as a candidate for clinical development.

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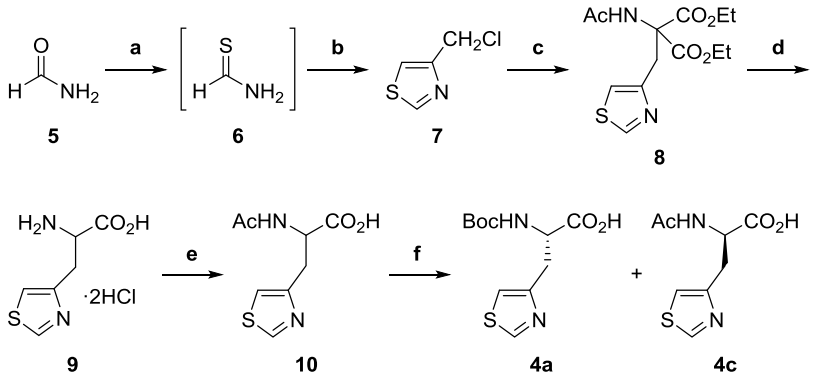
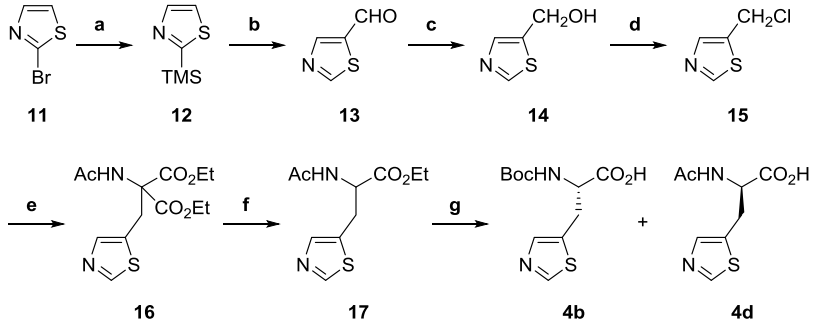
Scheme 2. Synthesis of *N*-Boc-3-(Thiazol-4-yl)-L-alanine 3a[a]



[a]Reagents and conditions: (a) Lawesson's reagent, THF, 0 °C; (b) 1,3-dichloroacetone, THF, 0 °C, 63%; (c) diethyl acetamidomalonate, NaOEt, KI, EtOH, reflux, 89%; (d) conc. aq. HCl, reflux, 74%; (e) aq. NaOH, Ac_2O; and (f) 1) aminoacylase, 37 °C, pH 7.2; 2) Boc_2O, THF, rt, 29%.

Scheme 3. Synthesis of *N*-Boc-3-(Thiazol-5-yl)-L-alanine 4b[a]



[a]Reagents and conditions: (a) TMS-Cl, *n*-BuLi, Et_2O, −78 °C, 89%; (b) *N*-formylmorpholine, Et_2O, −78 °C, 50%; (c) $NaBH_4$, MeOH, rt, 61%; (d) $SOCl_2$ neat, rt, quant; (e) diethyl acetamidomalonate, NaOEt, KI, EtOH, reflux, 71%; (f) (1) aq. NaOH; (2) aq. HCl, reflux, 52%; and (g) (1) aq. NaOH; (2) aminoacylase, 37 °C, pH 7.3; (3) Boc_2O, THF, rt, 44%.

RESULTS AND DISCUSSION

Chemistry. *N-Terminus Fragments.* All N-terminus fragments **3a**−**k** were synthesized from readily available *N*-Cbz protected L-serine **2a**, L-threonine **2b**, and allo-L-threonine **2c** (Scheme 1).

N-Terminus amino acids, 2-oxooxazolidine-4-carboxylic acid **3a**, (4*S*,5*R*)- or (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acids **3b** and **3c**, were synthesized on the basis of previous reports[28−32] in one step by treatment of **2a**−**c** with sodium hydroxide, with oxazolidinone ring formation occurring rapidly. *N*-Methyl or *N*-benzyl (4*S*,5*R*)-5-methyl-2-oxooxazolidine-4-carboxylic acids **3g** and **3h** were both synthesized from carboxylic acid **3b**, via esterification with benzyl alcohol to afford benzyl ester **3d**. The benzyl ester **3d** was alkylated with iodomethane or benzyl bromide to afford *N*-methyl derivative **3e** or *N*-benzyl derivative **3f**, respectively. The benzyl ester **3e** was deprotected by catalytic hydrogenation to give *N*-methyl-4-carboxylic acid **3g**. On the other hand, the benzyl ester **3f** was deprotected with aqueous lithium hydroxide to give *N*-benzyl-4-carboxylic acid **3h**.

Trifluoromethanesulfonate **3k** was synthesized from carboxylic acid **3c** via esterification with thionyl chloride in methanol to afford methyl ester **3i**, which was reduced with sodium borohydride to give alcohol **3j**. The alcohol derivative **3j** was sulfonylated with trifluoromethanesulfonic anhydride to give trifluoromethanesulfonate **3k**.

Middle-Part Fragments. *N*-Boc-3-(thiazol-4-yl)-L-alanine **4a** and *N*-Boc-3-(thiazol-5-yl)-L-alanine **4b** were synthesized by alkylation of diethyl acetamidomalonate (Schemes 2 and 3).

N-Boc-3-(thiazol-4-yl)-L-alanine **4a** was synthesized on the basis of previous reports.[33−37] Here, we describe the synthesis of **4a** from readily available formamide **5** (Scheme 2). Formamide **5** was converted to thioformamide **6** with Lawesson's reagent. Thiazole ring formation was accomplished by treatment of thioformamide **6** with 1,3-dichloroacetone to afford 4-chloromethylthiazole **7**. The chloro derivative **7** was converted to the desired (thiazol-4-yl)-L-alanine **4a** via alkylation of diethyl acetamidomalonate to give **8**, with hydrolysis to provide unprotected (thiazol-4-yl)-DL-alanine **9** and acetylation to give **10**, which was not isolated. The acetyl derivative **10** was optically resolved by enzymatic deacetylation, with the amino group of the deacetylated L-enantiomer protected with Boc_2O to give L-isomer **4a**, with 99% ee.

Similarly, the synthesis of *N*-Boc-3-(thiazol-5-yl)-L-alanine **4b** was accomplished from readily available 2-bromothiazole **11** (Scheme 3). The intermediates 2-trimethylsilylthiazole **12** and 5-formylthiazole **13** were synthesized on the basis of Dondoni's method.[38] The formyl group of **13** was reduced with sodium borohydride to afford hydroxymethylthiazole **14**.[39−41] The hydroxy group of **14** was chlorinated with thionyl chloride to afford 5-chloromethylthiazole **15**. The chloro derivative **15** was converted to (thiazol-5-yl)-L-alanine **4b** via diethyl ester **16** and ethyl ester **17** in a manner similar to Scheme 2.

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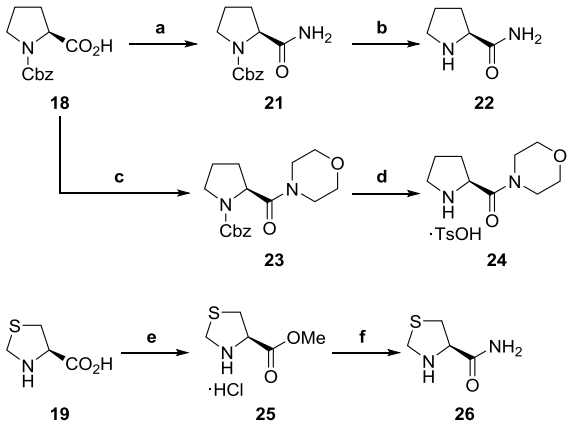
C-Terminus Fragments. All C-terminus fragments were synthesized from readily available *N*-Cbz-L-proline **18**, (4*R*)-4-thiazolidinecarboxylic acid **19** and *N*-Boc-L-proline **20** as starting materials on the basis of previous reports (Schemes 4 and 5).[21,42−44]

Scheme 4. Synthesis of L-Prolinamide 22, L-Prolylmorpholine *p*-Toluenesulfonate 24 and (4*R*)-4-Thiazolidinecarboxamide 26[a]



[a]Reagents and conditions: (a) (1) $ClCO_2Et$, Et_3N, THF, −25 °C; (2) aq. NH_3, THF, −25 °C, 87%; (b) H_2, Pd−C, MeOH, quant; (c) morpholine, HOBt, Et_3N, DCC, DMF, 0 °C to rt, 70%; (d) H_2, Pd−C, *p*-TsOH·H_2O, MeOH, rt, quant; and (e) $SOCl_2$, MeOH, rt, 99%; (f) liq. NH_3, rt, 78%.

L-Prolinamide **22**,[42] L-prolylmorpholine *p*-toluenesulfonate **24**, and (4*R*)-4-thiazolidinecarboxamide **26**[21] were synthesized in two steps from **18** and **19** as starting materials, respectively (Scheme 4).

Both (2*S*)-2-cyanopyrrolidine *p*-toluenesulfonate **29** and (2*R*)-2-methylpyrrolidine hydrochloride **33** were synthesized from **20** on the basis of previous reports[43,44] (Scheme 5).

TRH Mimetics. All fragments were coupled from the C-terminus to the N-terminus by a general peptide synthetic route to afford the TRH mimetics shown in Schemes 6 and 7.[45,46]

When **4a** and **4b** were used as the middle part, the synthetic route was as shown in Scheme 6. The C-terminus fragments **22**, **24**, **26**, **29**, and **33** were coupled with the middle-part fragments **4a** and **4b** using DCC and HOBt or HOSu to obtain *N*-Boc dipeptide mimetics **34**, **35**, **37**−**41**, **43**, and **44**, respectively. The Boc groups of intermediates **34**, **35**, **37**−**41**, **43**, and **44** were deprotected under acidic conditions (HCl in solvents, TFA, or *p*-toluenesulfonic acid monohydrate) to afford dipeptide mimetics **45**, **46**, **48**−**52**, **54**, and **55**, respectively. Intermediates **45**, **46**, **48**−**52**, **54**, and **55** were coupled with N-terminus fragments L-pyroglutamic acid, **3a**−**c**, to afford TRH mimetics **1**, **56**−**65**, **67**−**71**, and **73**−**77**, respectively.

When using commercially available N^{α}-Cbz-L-histidine hydrazide[47] as the middle-part fragment, the synthetic route was as shown in Scheme 7. The C-terminus fragments **22** and **33** were coupled with N^{α}-Cbz-L-histidine hydrazide by the azide method to afford *N*-Cbz dipeptide mimetics **36** and **42**, respectively. The Cbz group of intermediate **36** was deprotected with 25% hydrogen bromide in acetic acid to afford dipeptide **47**. On the other hand, the Cbz group of intermediate **42** was deprotected by catalytic hydrogenation to afford dipeptide mimetic **53**. Finally, dipeptide mimetics **47** and **53** were coupled with **3c** using DCC and HOSu to obtain TRH mimetics **66** and **72**, respectively.

The chemical structure of the TRH mimetics **1**, **56**−**74** are shown in Table 1. The yields of the *N*-Boc or *N*-Cbz dipeptide mimetics **34**−**44**, dipeptide mimetics **45**−**55**, and TRH mimetics **1**, **56**−**74** are shown in the Supporting Information.

The synthetic method to obtain TRH mimetics with carboxyl group **75** or secondary amide group **76** and **77** at the C-terminus is shown in Scheme 8.

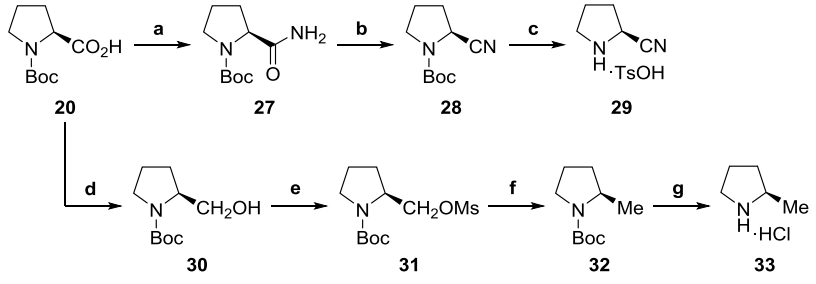
The synthetic method to obtain TRH mimetic with N-terminus 2-oxooxazolidine-4-yl-methyl moiety **78** is shown in Scheme 9.

The synthesized TRH mimetics **1** and **56**−**78** were purified by gel-filtration column chromatography packed with styrene-divinylbenzene copolymer resin (MCI GEL CHP-20P) using aqueous methanol as an eluent and/or silica gel column chromatography using chloroform−methanol(−water) as an eluent.

In the process of conversion from **45**−**47** and **49** having the L-prolinamide moiety or (4*R*)-4-thiazolidinecarboxamide moiety to TRH mimetics **1**, **56**−**66**, and **68**, diketopiperazines were produced as byproducts[7,21] (Scheme 10). The diketopiperazines were produced immediately after addition of triethylamine in the coupling reaction. On the other hand, in the case of **69**−**72** having the cyano or the methyl group, no diketopiperazines were produced.

Physicochemical Properties of TRH Mimetics. The physicochemical properties were estimated by clog *P* and Slog *P*. clog *P*, which is the log of the octanol/water partition coefficient, was calculated using ChemBioDraw Ultra. Slog *P*,

Scheme 5. Synthesis of (2*S*)-2-Cyanopyrrolidine *p*-Toluenesulfonate 29 and (2*R*)-2-Methylpyrrolidine Hydrochloride 33[a]



[a]Reagents and conditions: (a) (1) $ClCO_2Et$, Et_3N, THF, −25 °C; (2) aq. NH_3, THF, −25 °C, 86%; (b) $POCl_3$, imidazole, pyridine, 0 °C to rt, 90%; (c) (1) TFA, anisole, 0 °C; (2) *p*-TsOH·H_2O, quant; (d) BH_3-THF complex, THF, 0 °C, 98%; (e) MsCl, Et_3N, THF, 0 °C, 95%; (f) $NaBH_4$, DMSO, 80 °C, 46%; and (g) HCl in 1,4-dioxane, rt, quant.

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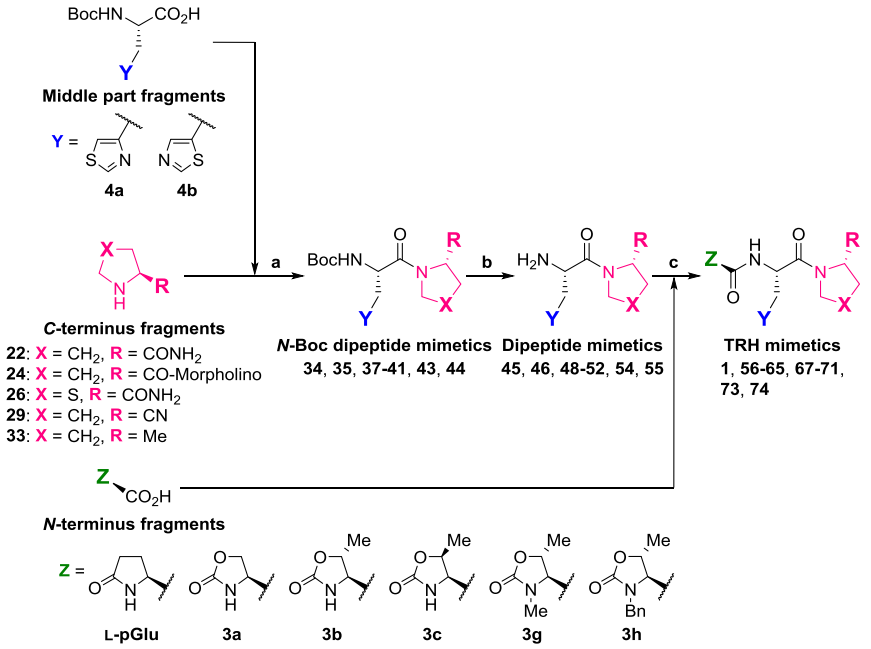
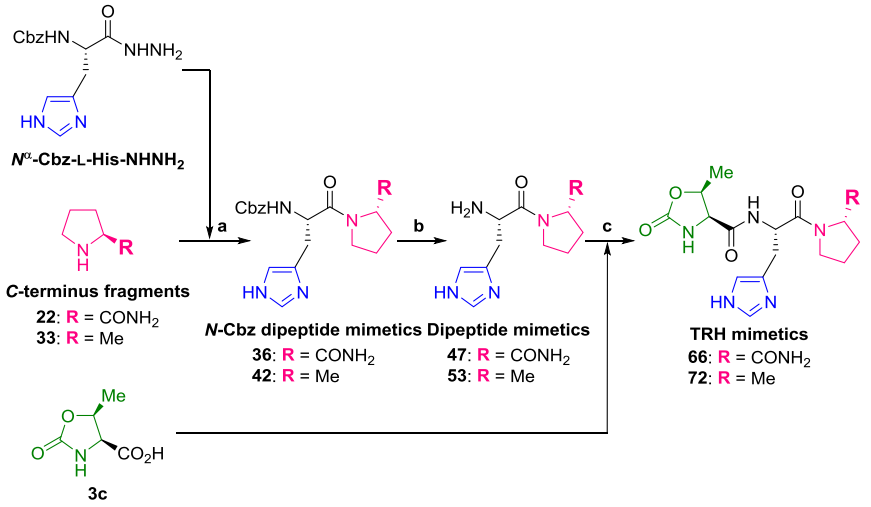
Scheme 6. General Synthetic Route of TRH Mimetics Including 4a and 4b as Middle-Part Fragments[a]



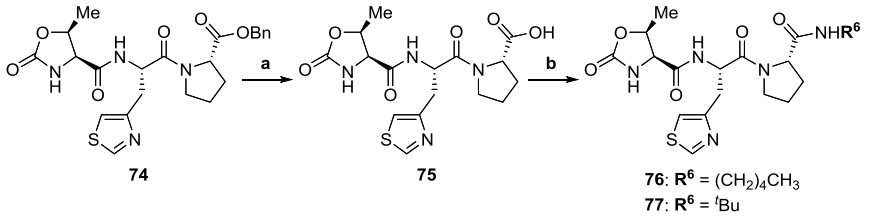
[a]Reagents and conditions: (a) DCC, HOBt or HOSu, DMF, 0 °C to rt; (b) HCl or TFA or *p*-TsOH·H_2O, 0−50 °C; and (c) DCC, HOSu, Et_3N, DMF, 0 °C to rt.

Scheme 7. General Synthetic Route of TRH Mimetics Including L-Histidine as the Middle-Part Fragment[a]



[a]Reagents and conditions: (a) (1) aq. HCl, $NaNO_2$, H_2O, 0 °C; (2) K_2CO_3, H_2O, 0 °C to rt or (1) HCl in 1,4-dioxane, isoamyl nitrite, DMF, −78 to 0 °C; (2) Et_3N, 0 °C to rt; (b) 25% HBr in acetic acid, rt or H_2, Pd−C, MeOH, rt; and (c) DCC, HOSu, Et_3N, DMF, 0 °C to rt.

Scheme 8. Synthesis of TRH Mimetics 75−77[a]



[a]Reagents and conditions: (a) LiOH·H_2O, MeOH−H_2O, rt, 80%; (b) amylamine or *tert*-butylamine, HOBt, DCC, DMF, 0 °C to rt, 62−65%.

which is the log of the octanol/water partition coefficient (including implicit hydrogens), was calculated using Molecular Operating Environment (MOE). This property is an atomic contribution model[48] obtained by calculating log *P* from the

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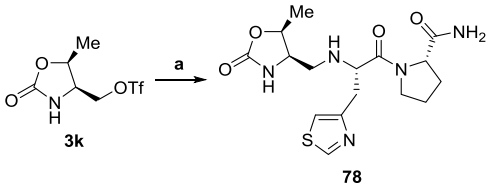
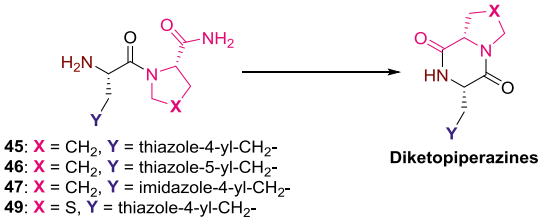
Scheme 9. Synthesis of TRH Mimetic 78[a]



[a]Reagents and conditions: (a) **45**, Et_3N, DMF, 0 °C to rt, 27%.

Scheme 10. Diketopiperazines Produced from Dipeptide Mimetics 45−47 and 49



given structure; that is, the correct protonation state (washed structures). Results may vary with the log *P* (o/w) descriptor. The training set for Slog *P* was ∼7000 structures. All results are shown in Table 1.

In Vivo CNS Effect of TRH Mimetics. We selected the reserpine-induced hypothermia model as the classical assay method for SAR including CNS effects in the first tier assay for compound selection. The CNS effects of TRH mimetics (**1**, **56**−**78**) were evaluated by their antagonistic effect on reserpine-induced hypothermia in mice.[14f,g] The mice used had rectal temperatures of 30 °C or lower about 18 h after subcutaneous administration of reserpine (3 mg/kg). Rectal temperature was measured by thermistor before and up to 7 h after, oral administration of TRH and TRH mimetics at a dose of 50 and 5 μmol/kg, respectively. The CNS effect of the test drugs on reserpine-induced hypothermia was evaluated on the basis of the area under the temperature−time curve after dosing (AUC_{0-7h}) (see the Experimental Section). All results are shown as the oral effective dose in Table 1. All animal studies were conducted under the approval of the Institutional Animal Care and Use Committees of Shionogi Research Laboratories. The results of the SAR study of the TRH mimetics are shown in Table 1.

We first conducted optimization from the N-terminus, examining TRH mimetics **1**, **58**−**61**, **64**, and **78**. The L-pyroglutamate of **1** was converted to one having the 2-oxooxazolidine carbonyl moiety **58**. The biological activity was maintained by introducing an oxygen atom (data not shown). Introducing a methyl group on the 5-position of oxazolidinone ring, the lipophilicity was increased (clog *P* = −0.528, Slog *P* = −0.856). The CNS effects vary depending on stereochemistry. The cis configuration **64** had a higher effect than the trans configuration **59**. Introducing a methyl group **60** or a benzyl group **61** on the 3-position of the oxazolidinone ring decreased the CNS effects (data not shown). Therefore, the *NH* of the 3-position of the oxazolidinone ring was essential for the high activity. Converting the carbonyl group **64** to methylene **78** increased the lipophilicity (clog *P* = −1.097, Slog *P* = −0.383), but the CNS effect decreased slightly. As a result, (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carbonyl moiety **64** was considered to be the best for the N-terminus.

Next, we optimized the middle part of the mimetics. For the middle part, when the 3-(thiazol-4-yl)-L-alanine moiety **64** was introduced in place of L-histidine moiety **66**, **64** had a higher CNS effect than **66**. Increasing the lipophilicity seemed to lead to higher oral absorption and brain penetration. The substitution position on the thiazole ring was significantly important. The lipophilicity of the 3-(thiazol-4-yl)-L-alanine moiety **64** and the 3-(thiazol-5-yl)-L-alanine moiety **65** were equal (clog *P* = −0.528, Slog *P* = −0.856); however, the biological activity of **65** was much lower than that of **64**. This indicated that the positioning of the nitrogen and sulfur atoms in the thiazole ring was critical for the activity.[49−51] As a result, the 3-(thiazol-4-yl)-L-alanine moiety **64** seemed to be the best for the middle part.

Finally, we optimized the C-terminus of the mimetics by replacing the L-prolinamide moiety with other moieties to increase the lipophilicity to improve the CNS effect, with TRH mimetics **67**−**70** and **73**−**77**. As for the C-terminus part of TRH mimetics **67**−**70**, **73**−**77** were examined. It is known that the L-prolinamide moiety is important for the formation of the hydrogen bond with Arg283 of TRH receptor[52,53] to have expression of the biological activities. In the case of the TRH mimetic having the (4*R*)-4-thiazolidinecarboxamide moiety **68**, both lipophilicity (clog *P* = 0.131, Slog *P* = −0.946) and CNS effect increased. When the L-prolinamide moiety was converted to L-proline secondary amide moiety **76** and **77** or tertiary amide moiety **67**, the lipophilicities increased; however, the CNS effects decreased remarkably. On the other hand, when L-prolinamide moiety **64** was converted to the (2*S*)-cyanopyrrolidine moiety **69**, lipophilicity (clog *P* = −0.0044, Slog *P* = 0.182) increased but the CNS effect decreased slightly. However, replacement of the L-prolinamide moiety of **64** with the (2*R*)-methylpyrrolidine moiety **70** led to much higher lipophilicity (clog *P* = 0.639, Slog *P* = 0.678) and CNS effect than **64**. When L-prolinamide moiety **64** was converted to L-prolinol moiety **73** and L-proline moiety **75**, the lipophilicities (**73**: clog *P* = −0.143, Slog *P* = −0.349, **75**: clog *P* = 0.331, Slog *P* = −0.257) increased but the CNS effects decreased markedly. TRH mimetics **56**, **57**, **62**, **63**, **71**, **72**, and **74** were synthesized and evaluated; however, the CNS effects of these compounds were less than that of **64**. As a result, L-prolinamide moiety **64**, (4*R*)-4-thiazolidinecarboxamide moiety **68**, (2*S*)-cyanopyrrolidine moiety **69**, and (2*R*)-methylpyrrolidine moiety **70** were considered to be suitable for the C-terminus part.

TRH Receptor Binding in Rat. TRH receptor binding of TRH and TRH mimetics were evaluated using a rat brain preparation. As the TRH receptor included both TRH-R1 and TRH-R2 in the rat brain preparation, it was not possible to establish whether TRH mimetics were binding preferentially to TRH-R1 or TRH-R2. The results of the TRH receptor binding assay are shown in Table 1.

TRH mimetics **1**, **58**, **59**, **64**−**66**, and **68**, which have L-prolinamide moiety or (4*R*)-4-thiazolidinecarboxamide moiety at the C-terminus indicated much higher affinity than that of TRH mimetics, which have (2*S*)-cyanopyrrolidine moiety **69**, (2*R*)-methylpyrrolidine moiety **70**, (2*S*)-hydroxymethylpyrrolidine moiety **73**, and L-proline moiety **75**. When the C-terminus (2*R*)-methylpyrrolidine moiety **70** was oxidized to (2*S*)-hydroxymethylpyrrolidine moiety **73** and L-proline moiety **75**, the affinities to TRH receptor decreased. As for the middle part, the TRH mimetic, which has 3-(thiazol-4-yl)-L-alanine moiety **64**, had a higher affinity than L-histidine

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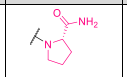
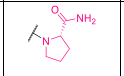
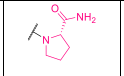
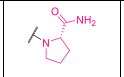
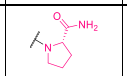
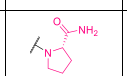
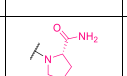
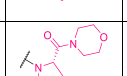
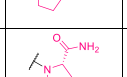
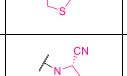
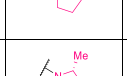
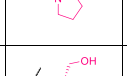
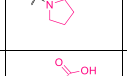
Table 1. SAR Study of TRH Mimetics on Physicochemical Properties, CNS Effects, and Rat TRH Receptor Binding Affinities

compounds	Z	Y	X and R	clog P[a]	Slog P[b]	oral effective dose[c] (nmol/°C/kg)	TRH-R binding[d] K_i (nM)
TRH				-2.792	-1.808	941 ± 168	25
1				-2.047	-1.075	19 ± 5	5.4
58				-1.047	-1.245	-[e]	12
59				-0.528	-0.856	214 ± 28	56
64				-0.528	-0.856	23 ± 3	3.5
65				-0.528	-0.856	63 ± 7	> 720
66				-1.273	-1.590	32 ± 8	23
67				0.284	-0.483	1037 ± 325	-[f]
68				0.131	-0.946	13 ± 1	19
69				-0.0044	0.182	47 ± 13	354
70				0.639	0.678	17 ± 2	246
73				-0.143	-0.349	71 ± 15	461
75				0.331	-0.257	90 ± 18	> 769
76				1.854	0.965	64 ± 11	-[f]
77				0.975	0.573	290 ± 54	-[f]
78				-1.097	-0.383	43 ± 8	-[f]

[a]clog *P* was calculated using ChemBioDraw Ultra. [b]Slog *P* was calculated using MOE. [c]Effective dose for increasing rectal body temperature by 1 °C per h (nmol/°C/kg body weight) in reserpine-induced hypothermia mice. Each value represents the mean ± SD of at least three animals. The formula for computation is as follows: AUC_{0-7h}: area under the rectal temperature−time curve for 7 h, Administration dose (5 or 50 μmol/kg)/((AUC_{0-7h} of test compounds − temperature at time 0 × 7 h of test compounds) − (AUC_{0-7h} of saline temperature at time 0 × 7 h of saline)/7 h). [d]K_i values were obtained from the inhibition to [^{3}H]-(3-Me-His^2)-TRH using a membrane preparation of the rat whole brain. [e]No data. [f]Not assayed.

moiety **66**. The TRH mimetic with 3-(thiazol-5-yl)-L-alanine moiety **65** did not show any affinity at all. Therefore, the positioning of the nitrogen and sulfur atoms in the thiazole ring was critical not only for the CNS effect but also for binding to TRH receptor. On the other hand, L-histidine moiety **66** showed the same affinity as TRH. As for the N-terminus part, 2-oxooxazolidine carbonyl moiety **58** had almost equal affinity to L-pyroglutamate moiety **1**. The cis configuration **64** between the methyl group and the carbonyl group had much higher affinity than the trans configuration **59**. It is considered that the CNS effects among TRH mimetics are not equal to the binding affinity to TRH receptor.

As a result of their CNS effect in mice, physicochemical properties, and TRH receptor binding affinity, four TRH mimetics **64**, **68**−**70** were selected for further investigation of PK parameters in rats.

*PK Properties of TRH Mimetics **64**, **68**−**70** in Rat.* The clearance (CL), half-life times ($t_{1/2}$), volume of distribution at steady state (Vd_{ss}), and mean residence times (MRT) of the four TRH mimetics **64**, **68**−**70** in rat after intravenous (i.v.) administration at a dose of 1 μmol/kg were evaluated. Also, the bioavailability (BA) of these four TRH mimetics in rat after per oral administration was evaluated. Moreover, free fraction ratio (fu) in serum was evaluated in vitro examinations. These PK properties are shown in Table 2. The CL of four TRH mimetics was lower than that of TRH. However, the $t_{1/2}$ and MRT of four TRH mimetics which have L-prolinamide moiety **64**, (4*R*)-4-thiazolidinecarboxamide moiety **68**, (2*S*)-cyano-pyrrolidine moiety **69**, and (2*R*)-methylpyrrolidine moiety **70** at the C-terminus were much longer than that of TRH; TRH mimetics **64** and **68** were shorter than those for the TRH mimetics **69** or **70**. Although the Vd_{ss} of TRH mimetics **64**, **68**, and **69** were lower than that of TRH, TRH mimetic **70** showed the highest value. Therefore, the (2*R*)-methylpyrrolidine moiety is significant to increase the tissue distribution. The BA of TRH mimetics **64** and **70** were 1.1 and 4.7%, respectively. Oral BA for TRH mimetics **68** and **69** could not be calculated due to low absorption. As a consequence, TRH mimetic **70** had the best total PK properties among the four TRH mimetics and a favorable effect would be predicted in rats.

From the data of the CNS effect, PK properties, TRH receptor affinity, and the synthetic viewpoint that the diketopiperazine was not produced in the process of the coupling reaction, we selected TRH mimetic **70** as a candidate for further development. Moreover, in the process of scale-up synthesis of **70**, the trihydrate of **70** was obtained as a stable crystalline solid. We selected this trihydrate **79** (rovatirelin hydrate)[27,55,56] as the preferred candidate (Figure 3).

*X-ray Crystallography of **79**.* Single crystals of **79** were recrystallized by gradual cooling of a saturated aqueous solution and obtained as the trihydrate. **79** has four asymmetric carbons; their absolute stereochemistry was determined by X-ray structural analysis. The X-ray crystal structure is shown in Figure 4. Three water molecules of **79** were omitted to compare the X-ray crystal structure of TRH,[57] in which tartrate molecule and water molecules were omitted. **79** has three

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Table 2. PK Properties of TRH and TRH Mimetics 64, 68−70 in Rat Plasma[a]

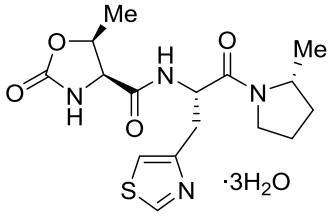
compounds	CL (mL/min/kg)	$t_{1/2}$ (min)[b]	Vd_{ss} (L/kg)	MRT (min)[c]	BA (%)[d]	fu (%)[e]
TRH	89.2	3.0	0.33	3.7	NC[f]	NC[f]
64	13.0	13.5	0.19	14.3	1.1	>99
68	14.2	9.8	0.16	11.0	NC[f]	>99
69	17.4	26.2	0.25	14.6	NC[f]	>99
70	30.6	21.6	0.58	19.1	4.7	>99

[a]Each value represents the mean of two animals. [b]$t_{1/2}$ of the plasma concentration ($C_{p,iv}$) of TRH mimetic after intravenous administration to rats were calculated by interpolation for plasma concentration of terminal phase. [c]MRT for the plasma concentration ($C_{p,iv}$) of TRH mimetic after intravenous administration to rats was calculated as defined in eq 1 by numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation.[54] [d]BA values of TRH mimetic after oral administration were calculated as defined in eq 2 using the plasma concentration after intravenous ($C_{p,iv}$) and oral administrations ($C_{p,po}$).

$$\mathrm{MRT} = \frac{\int_0^\infty t \times C_{p,iv}(t)\mathrm{d}t}{\int_0^\infty C_{p,iv}(t)\mathrm{d}t} \quad (1)$$

$$\mathrm{BA} = \frac{\text{dose, iv} \times \int_0^\infty C_{p,po}(t)\mathrm{d}t}{\text{dose, po} \times \int_0^\infty C_{p,iv}(t)\mathrm{d}t} \quad (2)$$

[e]fu: free fraction ratio in serum (%). [f]NC: not calculated.



79 (Rovatirelin Hydrate)

Figure 3. Chemical structure of **79** (rovatirelin hydrate).

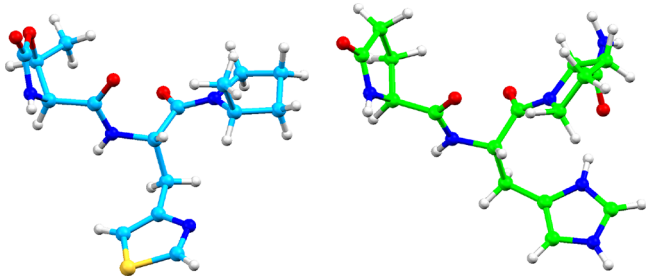


Figure 4. X-ray crystal structures of **79** (CCDC-1442496) (left) and TRH (CCDC-1275682) (right).[57] Crystallographic data are summarized in Tables S4−S9.

heterocyclic rings (oxazolidinone ring, thiazole ring, and pyrrolidine ring) and these groups are oriented to form a "propeller-shaped" conformation. This conformation is similar to the proposed TRH bioactive conformation.[58,59] Moreover, the "propeller-shaped" conformation seemed to be extremely important for high receptor binding. Three water molecules of **79** were coordinated to the amide bond, thiazole ring, and oxazolidinone ring. Each of them was used to form intramolecular hydrogen bonding (see the Supporting Information).

In vitro binding activities of TRH and **79** for human TRH-R have been reported previously.[56] The K_i values of TRH and **79** for human TRH-R are 128 and 702 nM, respectively. As the human TRH-R is more similar to TRH-R1 than TRH-R2,[12,13] the effect of TRH analogues may be related to TRH-R1 in human. The homology of the TRH-R1 receptor is also well conserved in many species. The human TRH-R is 90.3 and 89.2% homologous to that of mouse and rat at the DNA level, respectively.[11] Thus, the pharmacological effects of **79** in mouse and rat may be explained by the human TRH-R binding activity.

In Vivo CNS Effects of ***79****.* The CNS effects of TRH and **79** were tested by examining their in vivo antagonistic activity on reserpine-induced hypothermia in mice.[14] A representative figure for the rectal temperature−time curve is shown in Figure 5. After oral administration of **79** at a dose of 5 μmol/kg, the rectal temperature was maintained sustainably over a 2.5 °C temperature range from 2 to 7 h and compared with saline levels. TRH, at a dose of 50 μmol/kg, led to almost the same low rectal temperature profiles as the saline levels from 0 to 7 h after oral administration. The area under the rectal temperature−time curve up to 7 h (AUC) of **79** was higher than that

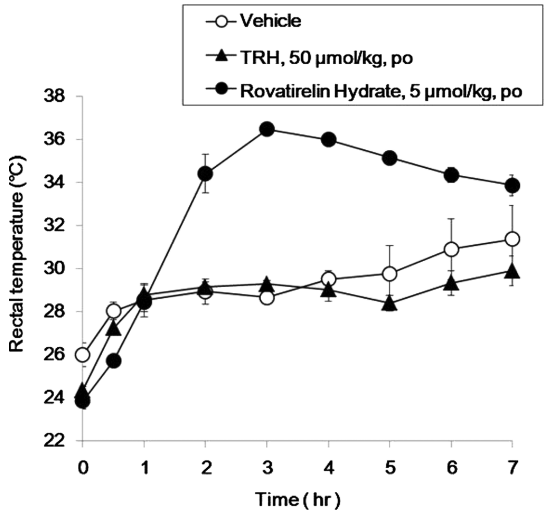


Figure 5. Rectal temperature−time profiles in reserpine-induced hypothermia mice after oral administration of TRH and **79** at a dose of 50 and 5 μmol/kg, respectively. Each point represents the mean ± SD of at least four animals.

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of saline and TRH (Table 3). The saline-stripped AUC (ΔAUC) of **79** was higher than that of TRH in spite of a 10-fold dose difference (5 vs 50 μmol/kg).

Table 3. Area under the Rectal Temperature−Time Curve after Oral Administration to Reserpine-Induced Hypothermia Mice of TRH and 79 at a Dose of 50 and 5 μmol/kg, Respectively[a]

	rectal temperature	
compounds (dose)	AUC_{0-7h} (°C·h)[b]	ΔAUC_{0-7h} (°C·h)[c]
vehicle	205.4 ± 11.1	
TRH (50 μmol/kg)	201.4 ± 3.3	−4.0 ± 3.3
79 (5 μmol/kg)	233.4 ± 4.3	28.0 ± 4.3

[a]Each value represents the mean ± SD of at least three animals. [b]AUC_{0-7h} was calculated by using the trapezoidal method. [c]ΔAUC = AUC_{0-7h}(compounds) − AUC_{0-7h}(saline).

*Improvement Effect of **79** on Cerebellar Ataxia in Rats.* We investigated improvement effects of **79** on ataxia in animals with cerebellar ataxia induced by administration of cytosine arabinoside (Ara-C) in the neonatal period. Neonate Sprague-Dawley rats were subcutaneously injected 60 mg/kg at age of 2 and 3 days (the day of confirming delivery: age of 0 day). Ara-C administered rats were subjected to the experiment at age of 4 weeks. **79** and TRH were orally administered once daily for 7 days to assess ataxia at 24 h after final administration. Severity of ataxia was assessed by the open field test which measured the number of ambulation events and falls. In brief, each animal was placed on the center of the circular open field (75 cm in diameter, 25 parts). The number of crossing parts (number of ambulation) and the number of falls for 3 min were measured to calculate the fall index ([number of falls]/[number of ambulation]). On the basis of the fall index, the severity of ataxia was assessed. The fall index in the control group was 2.27 (mean). In the **79** 0.1 mg/kg group, no significant decrease was observed in the fall index. In the **79** 0.3 mg/kg group, a significant decrease to 1.36 was observed in the fall index. The decrease in the fall index was dose-dependent up to 3 mg/kg. Although significant decrease was observed in the fall index in the **79** 10 and 30 mg/kg groups compared with that in the control group, the fall index increased more than the 3 mg/kg group. On the other hand, after administration of TRH, no significant decrease was observed in the fall index at any doses (30, 100, and 300 mg/kg). When TRH was intravenously administered once to assess ataxia at 30 min post-dose, a significant decrease was observed in the fall index at the doses of 10 and 30 mg/kg [fall index: mean ± SE (n = 10−17), vehicle: 2.06 ± 0.09, 10 mg/kg: 1.05 ± 0.22, and 30 mg/kg: 0.87 ± 0.17] (Figure 6).

*TSH Releasing Activity of **79**.* We examined the TSH releasing activity of **79** after oral administration in rats. After oral administration of **79** at a dose of 0.1 and 1 mg/kg, the plasma TSH levels at 0.5 h elevated up to 46.8 and 80.4 ng/mL, respectively. Then, plasma TSH level recovered to vehicle levels at 24 h (Figure 7).

*PK Properties of **79**.* Plasma concentration−time profiles of TRH and **79** after oral administration to fasted conscious rats at a dose of 40 mg/kg are presented in Figure 8. The PK parameters are summarized in Table 4. After oral administration, the profiles of plasma concentration of **79** were higher than those of TRH up to the experiment period (8 h) and, C_{max} and AUC_{0-8h} values of **79** were over 12 times higher

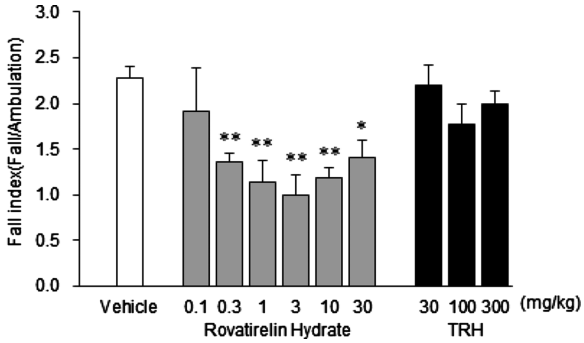

Figure 6. Improvement effects of 7 day repeated oral administration of **79** and TRH on ataxia in rats with Ara-C-induced cerebellar damage (age of 4 weeks). Data are expressed as mean ± SE (n = 10−18). *p < 0.05, **p < 0.01 vs vehicle group (Dunnett's test).

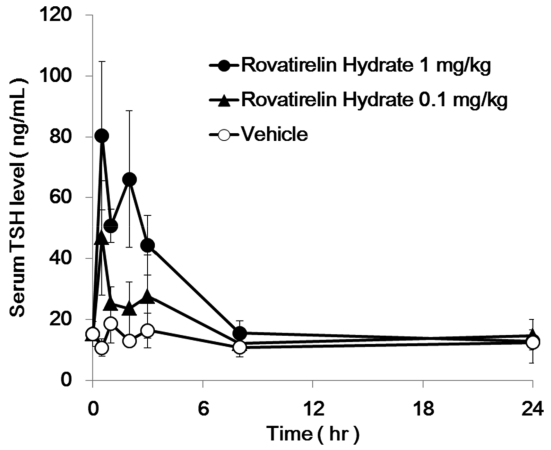

Figure 7. Plasma TSH level−time profiles after oral administration of **79** to male rats at a dose of 0.1 and 1.0 mg/kg, respectively. Each point represents the mean ± SD of at least four animals.

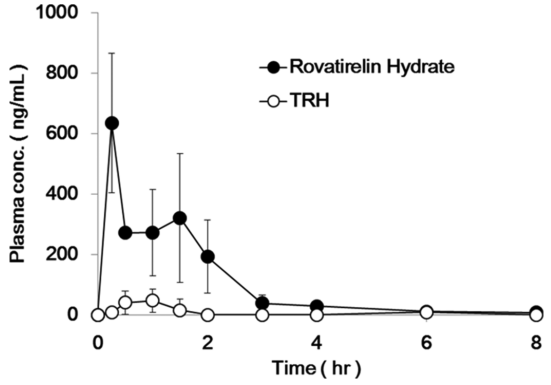

Figure 8. Plasma concentration−time profiles of TRH and **79** after oral administration to conscious rats at a dose of 40 mg/kg under fasted condition. Each point represents the mean ± SD of at least four animals.

than that of TRH. It seems that the stronger and more sustained efficacies of **79** for reserpine-induced hypothermia in mice and cerebellar ataxia in rats could also be attributed to the longer sustained plasma exposure and high brain Kp to **79** compared with TRH.

*Stability Study of **79**.* Stability of **79** in rat plasma and brain homogenate was evaluated. The amount of **79** remaining after incubation at 37 °C is shown in Table 5. Almost no

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Table 4. PK Parameters of TRH and 79 after Oral Administration to Fasted Conscious Rats at a Dose of 40 mg/kg[a]

compounds	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-8h} (ng·h/mL)[b]	brain Kp[c]
TRH	50 ± 39	0.75 ± 0.29	68 ± 72	ND[d]
79	636 ± 232	0.25 ± 0.00	817 ± 349	0.06 ± 0.03

[a]Each value represents the mean ± SD of at least four animals. [b]AUC_{0-8h} was calculated by using the trapezoidal method. [c]Brain Kp was calculated by the equation below.

$$\text{Brain Kp} = \text{ventral tegmental area } AUC_{0-12h}/\text{plasma } AUC_{0-12h}$$

Plasma and brain (ventral tegmental area) free concentration were determined after oral administration to rats at a dose of 40 mg/kg. [d]No data.

Table 5. Stability of 79 in Rat Plasma, Cerebrum, and Cerebellum Homogenate at 37 °C[a]

	compound remaining		
time (min)	plasma (%)	cerebrum (%)	cerebellum (%)
15	100 ± 0.1	100.2 ± 0.2	100 ± 0.0
30	99.8 ± 0.1	100.2 ± 0.1	99.9 ± 0.0
60	99.6 ± 0.3	100.3 ± 0.1	99.7 ± 0.0
120	99.8 ± 0.2	99.9 ± 0.1	99.6 ± 0.1
180	99.8 ± 0.1	99.7 ± 0.1	99.5 ± 0.1

[a]Values are mean ± SD of five experiments.

degradation of **79** was seen in rat plasma, cerebrum, and cerebellum homogenate samples even after 180 min. It was reported that taltirelin and TRH were degraded by the brain homogenate with $t_{1/2}$ of 64.4 and 7.9 min, respectively,[60] suggesting that **79** is more stable in the brain than taltirelin and TRH.

CYP Inhibition and Safety Information of ***79****.* **79** did not directly inhibit the 10 CYP enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5, and CYP4A11) examined by more than 50% at the highest concentration tested (300 μM). **79** caused no more than 10% inhibition of CYP activity except for CYP2C8, which displayed approximately 20% inhibition. The acute lethal dose of **79** in rats was over 2000 mg/kg in a single oral administration.

SUMMARY AND CONCLUSIONS

We conducted SAR studies of TRH to find orally effective TRH mimetics. The oxygen atom was introduced to the five-membered ring in the N-terminus part **58**, leading to better lipophilicity than L-pyroglutamate. Introduction of a methyl group to the 5-position of oxazolidinone ring **64** led to increased CNS effect and affinity to TRH receptor. The relative configuration between the methyl group and the carbonyl group was significant. Cis configuration **64** showed higher CNS effect and affinity to TRH receptor than trans configuration **59**. Moreover, the carbonyl group of **64** was important because when a methylene was introduced instead of carbonyl group **78**, the CNS effect decreased. It seemed that the carbonyl group was used to form a hydrogen bond with the target receptor or maintain the active conformation of the molecule. A thiazole ring in the middle-part, as in **1** and **64**, was more suitable than the imidazole ring, but the substitution position of the thiazole ring was significantly important. The 4-position substituent **64** showed high CNS effect, but the 5-position substituent **65** had no activity. The position of the nitrogen atom of the thiazole ring seemed to be important for forming a hydrogen bond with the target receptor and also for higher biological activity. It was speculated that converting the imidazole to thiazole lead to increasing the lipophilicity of molecule, with accompanying improvements in passing the blood–brain barrier. TRH mimetics that had the (4*R*)-4-thiazolidinecarboxamide moiety **68**, (2*S*)-cyanopyrrolidine moiety **69**, or the (2*R*)-methylpyrrolidine moiety **70** as the C-terminus part instead of the L-prolinamide moiety **64** maintained high biological activities. The L-prolinamide moiety was not essential for a high CNS effect. As a result of the SAR study, and taking into consideration the CNS effect, PK properties, and physicochemical characteristics, along with synthetic tractability, we selected **70** as a candidate, despite **70** having a weaker binding affinity for the TRH receptor than TRH and some of the TRH mimetics. Subsequently, TRH mimetic **70** was developed in clinical trials as the trihydrate **79** named rovatirelin hydrate.[55,56] Although rovatirelin hydrate showed approximately six times less in vitro binding activity for human TRH receptor than TRH,[56] in vivo efficacy of rovatirelin hydrate for reserpine-induced hypothermia in mice and on cerebellar ataxia in rats was stronger than that of TRH. The strong CNS effect of rovatirelin hydrate may be due to increased oral BA, higher distribution to the target brain region, and greater stability in plasma, cerebrum, and cerebellum than TRH. Rovatirelin hydrate was designed for higher lipophilicity than TRH for better intestinal and brain permeability. Therefore, rovatirelin hydrate, which was first found by our group,[55] is currently in a phase III clinical trial (NCT02889302) in SCD patients.

EXPERIMENTAL SECTION

General. All solvents and reagents were obtained from commercial sources and were used as received. Melting points (mp) were measured with a Yanagimoto melting point apparatus and were uncorrected. Infrared spectra were recorded on a Nicolet 20SXB FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were taken with a Varian VXR-200 or Gemini-200300 FT-NMR spectrometer using tetramethylsilane as an internal standard. Optical rotations were determined with a PerkinElmer 430 polarimeter. Elemental analyses were performed by Shionogi Research Laboratories (Shionogi & Co., Ltd. 3-1-1, Futaba-cho, Toyonaka-shi, Osaka 561-0825, Japan). For silica gel column chromatography, Kiesel gel 60 (0.063–0.20 mm, Merck) was employed for the purification. For gel-filtration chromatography, MCI GEL CHP-20P (75–150 g, Mitsubishi Chemical Industries) was utilized with aqueous MeOH as an eluent. Thin-layer chromatography (TLC) was carried out with Merck silica gel 603–254 plates mainly using the following three solvent systems: (a) $CHCl_3$/MeOH (9/1), (b) $CHCl_3$/MeOH/H_2O (32/6/0.5), and (c) $CHCl_3$/MeOH/H_2O (6/4/1). The spots were detected under ultraviolet irradiation at 254 nm and by the use of phosphomolybdic acid in ethanol solution and ninhydrin sprays.

General Procedure for the Synthesis of TRH Mimetics. *L-Pyroglutamyl-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**1**).* To an ice-cooled solution of L-pyroglutamic acid (1.76 g, 13.6 mmol) and HOSu (1.73 g, 15.0 mmol) in DMF (50.0 mL), DCC (3.09 g, 15.0 mmol) was added, and the mixture was stirred for 2 h at the same temperature. [3-

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(Thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**45**) (6.67 g, 15.0 mmol) and triethylamine (4.60 mL, 33.0 mmol) were added to the solution, and the mixture was stirred for 1 h. The ice bath was removed and the mixture was stirred overnight. The precipitate was filtered off and the filtrate was concentrated under reduced pressure. A small amount of water was added to the residue, and the precipitate was filtered off. The filtrate was purified by gel-filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H_2O/MeOH). The purified fractions were concentrated in vacuo and the residue was lyophilized to afford the title compound **1** (2.54 g, 49%) as a white amorphous powder.

IR (KBr): 3294, 1683, 1639, 1541, 1518, 1444, 1263 cm^{-1}.

^1H NMR (200 MHz, CD_3OD): δ 8.95 (d, J = 2.0 Hz, 1H), 7.43 and 7.34 (d each, J = 2.0 Hz, total 1H), 4.95 (t, J = 7.0 Hz, 1H), 4.42 and 4.34 (m each, total 1H), 4.17 (m, 1H), 3.80 (m, 1H), 3.60−3.10 (m, 3H), 2.50−1.80 (m, 8H).

^{13}C NMR (75 MHz, CD_3OD): δ 182.20, 182.10, 177.54, 176.74, 175.28, 175.19, 174.69, 172.64, 155.99, 154.06, 118.59, 118.31, 62.32, 62.14, 58.52, 58.47, 58.40, 53.36, 53.27, 53.20, 50.24, 48.54, 35.25, 33.94, 33.41, 31.32, 30.99, 30.94, 27.16, 27.09, 26.38, 23.66.

$[\alpha]_D^{24}$ −42.9° (c 1.0, MeOH).

Anal. Calcd for $C_{16}H_{21}N_5O_4S{\cdot}H_2O$: C, 48.35; H, 5.83; N, 17.62; S, 8.07. Found: C, 48.35; H, 5.85; N, 17.36; S, 8.31.

TLC R_f (b) 0.24, (c) 0.65.

In a similar manner, other TRH mimetics (**56**−**74**) were prepared.

*L-Pyroglutamyl-[3-(thiazol-5-yl)-L-alanyl]-L-prolinamide (**56**).* The condensation of L-pyroglutaminic acid (0.380 g, 2.93 mmol) and [3-(thiazol-5-yl)-L-alanyl]-L-prolinamide dihydrochloride (**46**) (1.00 g, 2.93 mmol) yielded the title compound **56** (1.00 g, 2.93 mmol) as a white amorphous powder.

IR (KBr): 3393, 3081, 1684, 1639, 1540, 1443, 1247 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.89 (s, 1H), 7.76 and 7.71 (s each, total 1H), 4.90 (dd, J = 9.3, 4.5 Hz, 1H), 4.42 and 4.26 (dd each, J = 8.4, 4.5 Hz, total 1H), 4.18 (dd, J = 8.7, 4.8 Hz, 1H), 3.74 (m, 2H), 3.50 (dd, J = 15.3, 4.5 Hz, 1H), 3.24 (dd, J = 15.3, 9.3 Hz, 1H), 2.50−1.80 (m, 8H).

^{13}C NMR (75 MHz, CD_3OD): δ 182.22, 182.06, 177.45, 176.67, 175.52, 174.84, 171.61, 171.50, 155.92, 155.76, 143.81, 143.64, 136.16, 135.51, 62.25, 62.07, 58.56, 58.47, 54.23, 54.14, 33.68, 31.44, 30.90, 30.80, 29.53, 27.30, 27.13, 26.46, 23.68.

$[\alpha]_D^{23}$ −53.6° (c 1.0, MeOH).

Anal. Calcd for $C_{16}H_{21}N_5O_4S{\cdot}1.6H_2O$: C, 47.07; H, 5.97; N, 17.15; S, 7.85. Found: C, 47.05; H, 5.92; N, 17.34; S, 7.72.

TLC R_f (b) 0.20, R_f (c) 0.61.

*3-{N-L-Pyroglutamyl-[3-(thiazol-4-yl)-L-alanyl]}-(4R)-4-thiazolidinecarboxamide (**57**).* The condensation of L-pyroglutaminic acid (0.646 g, 5.00 mmol) and [3-(thiazol-4-yl)-L-alanyl]-(4R)-4-thiazolidinecarboxamide dihydrochloride (**49**) (1.65 g, 4.00 mmol) yielded the title compound **57** (0.783 g, 39%) as a white amorphous powder.

IR (KBr): 3301, 2936, 1685, 1518, 1419, 1330, 1262 cm^{-1}.

^1H NMR (200 MHz, CD_3OD): δ 8.95 (d, J = 1.8 Hz, 1H), 7.43 and 7.37 (d each, J = 1.8 Hz, total 1H), 5.05 (t, J = 6.8 Hz, 1H), 4.99 (d, J = 8.6 Hz, 1H), 4.86 (m, 1H), 4.45 (d, J = 8.6 Hz, 1H), 4.18 (dd, J = 8.6, 5.0 Hz, 1H), 3.50−3.10 (m, 4H), 2.50−1.90 (m, 4H).

$[\alpha]_D^{23}$ −87.6° (c 1.0, H_2O).

Anal. Calcd for $C_{15}H_{19}N_5O_4S_2{\cdot}1.2H_2O$: C, 42.99; H, 5.15; N, 16.71; S, 15.30. Found: C, 42.92; H, 5.07; N, 16.73; S, 15.18.

TLC R_f (b) 0.35.

*{[(4S)-(2-Oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-prolinamide (**58**).* The condensation of (4S)-2-oxooxazolidine-4-carboxylic acid (**3a**) (0.239 g, 1.82 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**45**) (0.932 g, 2.00 mmol) yielded the title compound **58** (0.368 g, 53%) as a white amorphous powder.

IR (KBr): 3294, 1752, 1676, 1637, 1542, 1519, 1446, 1407, 1237 cm^{-1}.

^1H NMR (200 MHz, CD_3OD): δ 8.95 (d, J = 2.0 Hz, 1H), 7.43 and 7.33 (d each, J = 2.0 Hz, total 1H), 4.96 (t, J = 7.2 Hz, 1H), 4.60−4.20 (m, 4H), 3.80 (m, 1H), 3.60−3.10 (m, 3H), 2.30−1.90 (m, 4H).

$[\alpha]_D^{25}$ −53.0° (c 1.0, H_2O).

Anal. Calcd for $C_{15}H_{19}N_5O_5S{\cdot}0.9H_2O$: C, 45.31; H, 5.27; N, 17.61; S, 8.06. Found: C, 45.27; H, 5.31; N, 17.65; S, 7.90.

TLC R_f (b) 0.22.

*{[(4S,5R)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-prolinamide (**59**).* The condensation of (4S,5R)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3b**) (0.264 g, 1.82 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**45**) (0.932 g, 2.00 mmol) yielded the title compound **59** (0.410 g, 57%) as a white amorphous powder.

IR (KBr): 3398, 1752, 1677, 1641, 1517, 1445, 1229 cm^{-1}.

^1H NMR (200 MHz, CD_3OD): δ 8.95 (d, J = 2.0 Hz, 1H), 7.43 and 7.33 (d each, J = 2.0 Hz, total 1H), 4.97 (t, J = 7.0 Hz, 1H), 4.60−4.30 (m, 2H), 3.93 (d, J = 5.4 Hz, 1H), 3.78 (m, 1H), 3.60−3.10 (m, 3H), 2.30−1.70 (m, 4H), 1.45 (d, J = 6.6 Hz, 3H).

$[\alpha]_D^{25}$ −30.3° (c 1.0, H_2O).

Anal. Calcd for $C_{16}H_{21}N_5O_5S{\cdot}0.9H_2O$: C, 46.68; H, 5.58; N, 17.01; S, 7.79. Found: C, 46.69; H, 5.62; N, 17.19; S, 7.93.

TLC R_f (b) 0.30.

*{[(4S,5R)-3,5-Dimethyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-prolinamide (**60**).* The condensation of (4S,5R)-3,5-dimethyl-2-oxooxazolidine-4-carboxylic acid (**3g**) (0.159 g, 1.00 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**45**) (0.535 g, 1.10 mmol) yielded the title compound **60** (0.201 g, 49%) as a white amorphous powder.

IR (KBr): 3412, 1751, 1678, 1519, 1544, 1519, 1437, 1401, 1237 cm^{-1}.

^1H NMR (200 MHz, CD_3OD): δ 8.96 (d, J = 2.0 Hz, 1H), 7.44 and 7.35 (d each, J = 2.0 Hz, total 1H), 5.04 (dd, J = 7.8, 6.2 Hz, 1H), 4.40 (m, 2H), 3.88 (d, J = 5.4 Hz, 1H), 3.80 (m, 1H), 3.60−3.10 (m, 3H), 2.67 (s, 3 H), 2.30−1.80 (m, 4H), 1.43 (d, J = 6.4 Hz, 3H).

$[\alpha]_D^{23}$ −31.9° (c 1.0, H_2O).

Anal. Calcd for $C_{17}H_{23}N_5O_5S{\cdot}1.4H_2O$: C, 46.97; H, 5.98; N, 16.11; S, 7.38. Found: C, 46.98; H, 5.91; N, 16.27; S, 7.37.

TLC R_f (b) 0.40.

*{[(4S,5R)-3-Benzyl-5-Methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-prolinamide (**61**).* The condensation of (4S,5R)-3-benzyl-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3h**) (0.235 g, 1.00 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**45**) (0.535 g, 1.10 mmol) yielded the title compound **61** (0.309 g, 64%) as a white amorphous powder.

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IR (KBr): 3412, 1752, 1679, 1644, 1543, 1516, 1442, 1415, 1227, 1206, 1092, 1066 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.99 (d, J = 2.1 Hz, 1H), 7.41 (d, J = 2.1 Hz, 1H), 7.50−7.10 (m, 5H), 4.95 (m, 1H), 4.73 (d, J = 15.3 Hz, 1H), 4.42 (m, 2H), 3.87 (d, J = 15.3 Hz, 1H), 3.78 (m, 1H), 3.68 (d, J = 5.1 Hz, 1H), 3.60−3.00 (m, 3H), 2.30−1.80 (m, 4H), 1.35 (d, J = 6.3 Hz, 3H).

$[\alpha]_D^{24.5}$ −43.7° (c 1.0, H_2O).

Anal. Calcd for $C_{23}H_{27}N_5O_5S{\cdot}1.2H_2O$: C, 54.47; H, 5.84; N, 13.81; S, 6.32. Found: C, 54.33; H, 5.86; N, 13.97; S, 6.30.

TLC R_f (b) 0.39.

*{[(4S,5R)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-5-yl)-L-alanyl]}-L-prolinamide (**62**).* The condensation of (4*S*,5*R*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3b**) (0.190 g, 1.29 mmol) and [3-(thiazol-5-yl)-L-alanyl]-L-prolinamide dihydrochloride (**46**) (0.440 g, 1.29 mmol) yielded the title compound **62** (0.220 g, 45%) as a white amorphous powder.

IR (KBr): 3397, 2979, 1753, 1677, 1639, 1527, 1446, 1403, 1230 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.89 (s, 1H), 7.76 and 7.70 (s each, total 1H), 4.92 (dd, J = 9.3, 3.9 Hz, 1H), 4.50−4.20 (m, 2H), 3.95 and 3.94 (d each, J = 5.1 Hz, total 1H), 3.90−3.60 (m, 2H), 3.50 (dd, J = 15.3, 3.9 Hz, 1H), 3.26 (dd, J = 9.3, 3.9 Hz, 1H), 2.30−1.80 (m, 4H), 1.46 and 1.45 (d each, J = 6.6 Hz, total 3H).

$[\alpha]_D^{23}$ −25.8° (c 1.0, MeOH).

Anal. Calcd for $C_{16}H_{21}N_5O_5S{\cdot}1.4H_2O$: C, 45.68; H, 5.70; N, 16.65; S, 7.62. Found: C, 45.63; H, 5.63; N, 16.82; S, 7.64.

TLC R_f (b) 0.22.

*3-{N-[(4S,5R)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(4R)-4-thiazolidinecarboxamide (**63**).* The condensation of (4*S*,5*R*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3b**) (0.528 g, 3.64 mmol) and [3-(thiazol-4-yl)-L-alanyl]-(4*R*)-4-thiazolidinecarboxamide dihydrochloride (**49**) (1.65 g, 4.00 mmol) yielded the title compound **63** (0.632 g, 42%) as a white amorphous powder.

IR (KBr): 3397, 2980, 2932, 1752, 1677, 1649, 1519, 1413, 1227 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.95 (d, J = 1.8 Hz, 1H), 7.43 and 7.36 (each d, J = 1.8 Hz, total 1H), 5.07 (t, J = 6.6 Hz, 1H), 4.98 (d, J = 8.7 Hz, 1H), 4.86 (m, 1H), 4.60−4.40 (m, 1H), 4.45 (d, J = 8.7 Hz, 1H), 3.95 (d, J = 5.1 Hz, 1H), 3.50−3.10 (m, 4H), 1.46 (d, J = 6.3 Hz, 3H).

$[\alpha]_D^{23}$ −58.8° (c 1.0, H_2O).

Anal. Calcd for $C_{15}H_{19}N_5O_5S_2{\cdot}1.1H_2O$: C, 41.58; H, 4.93; N, 16.16; S, 14.80. Found: C, 41.55; H, 4.92; N, 16.27; S, 14.82.

TLC R_f (b) 0.35.

*{[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-prolinamide (**64**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.264 g, 1.82 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**45**) (0.973 g, 2.00 mmol) yielded the title compound **64** (0.356 g, 50%) as a white amorphous powder.

IR (KBr): 3392, 2982, 1752, 1676, 1637, 1542, 1519, 1447, 1234 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.95 (d, J = 1.8 Hz, 1H), 7.43 and 7.35 (d each, J = 1.8 Hz, total 1H), 5.02 (t, J = 7.2 Hz, 1H), 4.90 (m, 1H), 4.38 (m, 1H), 4.33 (d, J = 8.7 Hz, 1H), 3.88 (m, 1H), 3.60−3.10 (m, 3H), 2.30−1.90 (m, 4H), 1.26 and 1.20 (d each, J = 6.6 Hz, total 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 176.45, 171.44, 170.21, 161.30, 154.99, 152.95, 117.48, 76.10, 61.23, 59.42, 52.08, 32.94, 30.30, 25.31, 22.70, 15.93, 15.79.

$[\alpha]_D^{26}$ −52.1° (c 1.0, H_2O).

Anal. Calcd for $C_{16}H_{21}N_5O_5S{\cdot}1.5H_2O$: C, 45.49; H, 5.73; N, 16.58; S, 7.59. Found: C, 45.53; H, 5.71; N, 16.85; S, 7.60.

TLC R_f (b) 0.35.

*{[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-5-yl)-L-alanyl}-L-prolinamide (**65**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.210 g, 1.47 mmol) and [3-(thiazol-5-yl)-L-alanyl]-L-prolinamide dihydrochloride (**46**) (0.500 g, 1.47 mmol) yielded the title compound **65** (0.270 g, 45%) as a white amorphous powder.

IR (KBr): 3406, 2982, 1752, 1677, 1638, 1542, 1447, 1404, 1343, 1300, 1237 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.90 and 8.89 (s each, total 1H), 7.77 and 7.73 (s each, total 1H), 5.00−4.90 (m, 2H), 4.41 (dd, J = 8.7, 4.8 Hz, 1H), 4.39 and 4.35 (d each, J = 8.7 Hz, total 1H), 3.91 (m, 1H), 3.71 (m, 1H), 3.50 (dd, J = 15.6, 3.6 Hz, 1H), 3.25 (dd, J = 15.6, 9.6 Hz, 1H), 2.30−1.80 (m, 4H), 1.26 and 1.18 (d each, J = 6.3 Hz, total 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 176.26, 170.47, 161.27, 154.66, 142.59, 135.04, 76.17, 76.01, 60.95, 59.45, 59.35, 53.17, 48.06, 32.58, 30.40, 28.54, 25.43, 22.75, 15.93, 15.78.

$[\alpha]_D^{25}$ −59.3° (c 1.0, H_2O).

Anal. Calcd for $C_{16}H_{21}N_5O_5S{\cdot}1.5H_2O$: C, 45.49; H, 5.73; N, 16.58; S, 7.59. Found: C, 45.39; H, 5.67; N, 16.75; S, 7.50.

TLC R_f (b) 0.29.

*[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-L-histidyl-L-prolinamide (**66**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (2.37 g, 16.3 mmol) and L-histidyl-L-prolinamide dihydrobromide (**47**) (7.53 g, 16.3 mmol) yielded the title compound **66** (0.340 g, 5.5%) as a white amorphous powder.

IR (KBr): 3282, 2984, 2882, 1749, 1675, 1636, 1543, 1448, 1343, 1235, 1097 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 7.64 and 7.60 (s each, total 1H), 6.96 and 6.90 (s each, total 1H), 5.00−4.80 (m, 1H), 4.41 (dd, J = 8.4, 4.2 Hz, 1H), 4.36 and 4.35 (d each, J = 8.4 Hz, total 1H), 3.85 (m, 1H), 3.43 (m, 1H), 3.12 and 2.98 (dd each, J = 14.4, 7.2 Hz, total 2H), 2.30−1.70 (m, 4H), 1.28 and 1.21 (d each, J = 6.6 Hz, total 3H).

$[\alpha]_D^{25}$ −48.4° (c 1.0, MeOH).

Anal. Calcd for $C_{16}H_{22}N_6O_5{\cdot}1.8H_2O$: C, 46.78; H, 6.28; N, 20.46. Found: C, 46.83; H, 6.27; N, 20.72.

TLC R_f (b) 0.12.

*1-{N-[(4S,5S)-5-Methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-prolylmorpholine (**67**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.300 g, 2.07 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolylmorpholine dihydrochloride (**48**) (0.850 g, 2.07 mmol) yielded the title compound **67** (0.560 g, 58%) as a white amorphous powder.

IR (KBr): 3411, 3284, 1760, 1638, 1518, 1445, 1233, 1112 cm^{-1}.

^1H NMR (200 MHz, CD_3OD): δ 8.98 and 8.94 (d each, J = 2.0 Hz, total 1H), 7.42 and 7.32 (d each, J = 2.0 Hz, total 1H), 5.09 (dd, J = 9.4, 4.6 Hz, 1H), 5.00−4.70 (m, 2H), 4.33 and 4.30 (d each, J = 8.6 Hz, total 1H), 4.10−3.50 (m, 10H), 3.38 (dd, J = 15.0, 4.6 Hz, 1H), 3.15 (dd, J = 15.0, 9.4 Hz, 1H), 2.40−1.60 (m, 4H), 1.21 and 1.17 (d each, J = 6.6 Hz, total 3H).

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$[\alpha]_D^{25}$ −45.8° (*c* 1.0, MeOH).

Anal. Calcd for $C_{20}H_{27}N_5O_6S\cdot1.4H_2O$: C, 48.95; H, 6.12; N, 14.27; S, 6.53. Found: C, 48.84; H, 6.13; N, 14.44; S, 6.43.

TLC R_f (b) 0.64.

*3-{N-[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(4R)-4-thiazolidinecarboxamide (**68**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.528 g, 3.64 mmol) and [3-(thiazol-4-yl)-L-alanyl]-(4*R*)-4-thiazolidinecarboxamide dihydrochloride (**49**) (1.65 g, 4.00 mmol) yielded the title compound **68** (0.704 g, 47%) as a white amorphous powder.

IR (KBr): 3309, 2986, 2937, 1752, 1678, 1650, 1519, 1415, 1333, 1231, 1097 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.99 and 8.95 (d each, *J* = 2.1 Hz, total 1H), 7.43 and 7.39 (d each, *J* = 2.1 Hz, total 1H), 5.11 (t, *J* = 6.6 Hz, 1H), 5.10 (d, *J* = 8.7 Hz, 1H), 5.00−4.70 (m, 2H), 4.48 (d, *J* = 8.7 Hz, 1H), 4.34 (d, *J* = 8.7 Hz, 1H), 3.50−3.10 (m, 4H), 1.22 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 174.23, 171.06, 170.26, 161.32, 155.21, 152.74, 117.62, 76.14, 64.06, 59.28, 52.08, 49.97, 33.55, 33.15, 15.86.

$[\alpha]_D^{25.5}$ −80.4° (*c* 1.0, MeOH).

Anal. Calcd for $C_{15}H_{19}N_5O_5S_2\cdot H_2O$: C, 41.75; H, 4.91; N, 16.23; S, 14.86. Found: C, 41.73; H, 4.94; N, 16.23; S, 14.82.

TLC R_f (b) 0.37.

*1-{N-[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2S)-2-cyanopyrrolidine (**69**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.210 g, 1.43 mmol) and 1-*N*-[3-(thiazol-4-yl)-L-alanyl]-(2*S*)-2-cyanopyrrolidine di-trifluoroacetate (**50**) (0.970 g, 1.43 mmol) yielded the title compound **69** (0.330 g, 61%) as a white amorphous powder.

IR (KBr): 3299, 2984, 2242, 1754, 1653, 1541, 1517, 1443, 1341, 1233, 1097 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.98 (d, *J* = 2.1 Hz, 1H), 7.35 (d, *J* = 2.1 Hz, 1H), 5.00−4.85 (m, 2H), 4.70 (dd, *J* = 7.8, 3.6 Hz, 1H), 4.39 and 4.34 (d each, *J* = 8.4 Hz, total 1H), 3.77 (m, 1H), 3.43 (m, 1H), 3.40−3.20 (m, 2H), 2.30−1.80 (m, 4H), 1.23 (d, *J* = 6.3 Hz, 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 171.34, 170.45, 161.35, 155.16, 152.38, 118.93, 117.75, 76.21, 59.23, 52.08, 47.42, 47.28, 33.15, 30.44, 25.69, 15.83.

$[\alpha]_D^{25}$ −35.0° (*c* 1.0, MeOH).

Anal. Calcd for $C_{16}H_{19}N_5O_4S\cdot1.1H_2O$: C, 48.38; H, 5.38; N, 17.63; S, 8.07. Found: C, 48.38; H, 5.39; N, 17.82; S, 7.94.

TLC R_f (b) 0.46.

*1-{N-[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2R)-2-methylpyrrolidine (**70**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (3.51 g, 24.2 mmol) and 1-*N*-[3-(thiazol-4-yl)-L-alanyl]-(2*R*)-2-methylpyrrolidine dihydrochloride (**51**) (7.56 g, 24.2 mmol) yielded the title compound **70** (5.25 g, 59%) as a white amorphous powder.

IR (KBr): 3279, 2972, 2877, 1763, 1655, 1626, 1548, 1456, 1384, 1231 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.97 and 8.96 (d each, *J* = 2.1 Hz, total 1H), 7.34 and 7.33 (d each, *J* = 2.1 Hz, total 1H), 5.19 and 5.05 (t each, *J* = 7.5 Hz, total 1H), 4.92 (dq, *J* = 8.7, 6.6 Hz, 1H), 4.37 and 4.35 (d each, *J* = 8.7 Hz, total 1H), 4.07 and 3.92 (m each, total 1H), 3.75 (m, 1H), 3.41 (m, 1H), 3.22 (m, 2H), 2.00−1.50 (m, 4H), 1.28 and 1.22 (d each, *J* = 6.6 Hz, total 3H), 1.21 and 1.02 (d each, *J* = 6.6 Hz, total 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 170.47, 170.33, 170.16, 169.83, 161.30, 155.04, 154.94, 152.97, 152.92, 117.33, 76.12, 76.05, 59.40, 59.33, 54.43, 54.17, 51.94, 51.82, 47.63, 46.11, 34.63, 33.54, 33.26, 32.20, 24.37, 21.86, 20.61, 18.87, 15.83.

$[\alpha]_D^{23}$ −4.9° (*c* 1.0, MeOH).

Anal. Calcd for $C_{16}H_{22}N_4O_4S\cdot H_2O$: C, 49.99; H, 6.29; N, 14.57; S, 8.34. Found: C, 49.83; H, 6.22; N, 14.83; S, 8.37.

TLC R_f (b) 0.56.

*1-{N-[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-5-yl)-L-alanyl}-(2R)-2-methylpyrrolidine (**71**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.140 g, 0.960 mmol) and 1-*N*-[3-(thiazol-5-yl)-L-alanyl]-(2*R*)-2-methylpyrrolidine dihydrochloride (**52**) (0.300 g, 0.960 mmol) yielded the title compound **71** (0.130 g, 37%) as a white amorphous powder.

IR (KBr): 3274, 3079, 2973, 2875, 1761, 1680, 1626, 1539, 1445, 1401, 1383, 1344, 1232 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.90 (s, 1H), 7.73 and 7.72 (s each, total 1H), 5.00−4.80 (m, 1H), 5.04 and 4.89 (t each, *J* = 7.5 Hz, total 1H), 4.40 and 4.37 (d each, *J* = 8.7 Hz, total 1H), 4.09 and 3.85 (m each, total 1H), 3.90−3.20 (m, 4H), 2.10−1.50 (m, 4H), 1.23 (d, *J* = 6.3 Hz, 3H), 1.25 and 1.09 (d each, *J* = 6.3 Hz, total 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 170.74, 170.53, 170.20, 170.01, 161.80, 155.35, 155.22, 143.22, 134.82, 76.54, 76.47, 59.75, 55.21, 54.88, 53.56, 53.35, 48.22, 33.67, 32.72, 30.54, 29.53, 24.85, 22.33, 21.11, 19.41, 16.24.

$[\alpha]_D^{22}$ −12.3° (*c* 1.0, MeOH).

Anal. Calcd for $C_{16}H_{22}N_4O_4S\cdot1.2H_2O$: C, 49.52; H, 6.34; N, 14.44; S, 8.26. Found: C, 49.39; H, 6.18; N, 14.69; S, 8.25.

TLC R_f (b) 0.50.

*1-{N-[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-L-histidyl}-(2R)-2-methylpyrrolidine (**72**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.600 g, 4.12 mmol) and 1-*N*-(L-histidyl)-(2*R*)-2-methylpyrrolidine (**53**) (1.00 g, 4.50 mmol) yielded the title compound **72** (0.420 g, 29%) as a white amorphous powder.

IR (KBr): 3398, 3271, 2975, 2877, 1749, 1626, 1541, 1449, 1385, 1345, 1232 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 7.63 and 7.62 (d each, *J* = 1.2 Hz, total 1H), 6.88 (s, 1H), 5.00−4.85 (m, 1H), 5.04 and 4.89 (t each, *J* = 7.5 Hz, total 1H), 4.37 and 4.35 (d each, *J* = 8.7 Hz, total 1H), 4.08 and 3.86 (m each, total 1H), 3.80−3.20 (m, 2H), 3.02 and 2.93 (dd each, *J* = 14.7, 7.5 Hz, total 1H), 2.10−1.50 (m, 4H), 1.22 (d, *J* = 6.6 Hz, 3H), 1.27 and 1.04 (d each, *J* = 6.6 Hz, total 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 171.28, 171.12, 170.58, 170.24, 161.80, 136.63, 136.49, 134.59, 117.86, 76.61, 76.54, 59.79, 59.72, 54.71, 54.58, 52.74, 52.69, 48.01, 46.46, 33.59, 32.63, 31.66, 30.53, 24.77, 22.31, 20.91, 19.25, 16.23.

$[\alpha]_D^{21}$ −7.4° (*c* 1.0, MeOH).

Anal. Calcd for $C_{16}H_{23}N_5O_4\cdot1.3H_2O$: C, 51.55; H, 6.92; N, 18.79. Found: C, 51.57; H, 6.72; N, 18.86.

TLC R_f (b) 0.21.

*1-{N-[(4S,5S)-5-Methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-Prolinol (**73**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.299 g, 1.00 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinol dihydrochloride (**54**) (0.101 g, 1.00 mmol) yielded the title compound **73** (0.168 g, 44%) as a white amorphous powder.

IR (KBr): 3276, 1750, 1627, 1542, 1518, 1450, 1407, 1385, 1342, 1231, 1097 cm^{-1}.

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^{1}H NMR (300 MHz, CD_3OD): δ 8.98 and 8.95 (d each, J = 2.1 Hz, total 1H), 7.36 and 7.35 (d each, J = 2.1 Hz, total 1H), 5.21 and 5.06 (t each, J = 7.5 Hz, total 1H), 4.91 (m, 1H), 4.37 and 4.35 (d each, J = 8.7 Hz, total 1H), 4.06 (m, 1H), 3.90−3.70 (m, 1H), 3.51 (dd, J = 10.8, 3.9 Hz, 1H), 3.43 (dd, J = 10.8, 6.3 Hz, 1H), 3.40 (m, 1H), 3.25 (m, 2H), 2.00−1.60 (m, 4H), 1.25 and 1.22 (d each, J = 6.3 Hz, total 3H).

$[\alpha]_D^{25}$ −10.7° (c 0.50, H_2O).

Anal. Calcd for $C_{16}H_{22}N_4O_5S{\cdot}1.5H_2O$: C, 46.93; H, 6.15; N, 13.68, S, 7.83. Found: C, 46.97; H, 6.20; N, 13.66; S, 7.65.

TLC R_f (b) 0.37.

*{[(4S,5S)-5-Methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-proline Benzyl Ester (**74**).* The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**3c**) (0.316 g, 2.17 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-proline benzyl ester dihydrochloride (**55**) (0.865 g, 2.17 mmol) yielded the title compound **74** (0.496 g, 47%) as a white amorphous powder.

IR (KBr): 3363, 2632, 2550, 1746, 1728, 1685, 1653, 1412, 1224, 1156, 1060 cm^{-1}.

^{1}H NMR (200 MHz, CD_3OD): δ 8.93 (d, J = 2.0 Hz, 1H), 7.35 (m, 5H), 7.30 (d, J = 2.0 Hz, 1H), 5.15 (s, 2H), 5.11 (m, 1H), 4.90 (m, 1H), 4.49 (m, 1H), 4.31 (d, J = 8.6 Hz, 1H), 3.90 (m, 1H), 3.60 (m, 1H), 3.17 (m, 2H), 2.25 (m, 1H), 1.97 (m, 3H), 1.17 (d, J = 6.6 Hz, 3H).

$[\alpha]_D^{26}$ −55.9° (c 0.51, MeOH).

Anal. Calcd for $C_{23}H_{26}N_4O_6S{\cdot}0.4H_2O$: C, 55.95; H, 5.47; N, 11.35; S, 6.49. Found: C, 55.83; H, 5.53; N, 11.52; S, 6.48.

TLC R_f (b) 0.62.

*{[(4S,5S)-5-Methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-proline (**75**).* To a solution of {[(4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-proline benzyl ester (**74**) (1.99 g, 4.09 mmol) in MeOH (10.0 mL)−H_2O (10.0 mL), lithium hydroxide hydrate (0.858 g, 20.5 mmol) was added and stirred for 1 h at room temperature. To the mixture, 1 M aqueous hydrochloric acid solution (20.5 mL, 20.5 mmol) was added and concentrated to about half volume under reduced pressure. To the mixture, ethyl acetate (50.0 mL) was added. After separation, the aqueous layer was washed with ethyl acetate (50.0 mL × 2) and then concentrated in vacuo. The residue was purified by gel-filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H_2O/MeOH). The purified fractions were concentrated in vacuo, and the residue was lyophilized to afford the title compound **75** (1.29 g, 80%) as a white amorphous powder.

IR (KBr): 3398, 3299, 1749, 1636, 1523, 1450, 1230 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ 8.98 and 8.95 (d each, J = 2.1 Hz, total 1H), 7.40 and 7.33 (d each, J = 2.1 Hz, total 1H), 5.09 (dd, J = 8.4, 5.4 Hz, 1H), 4.90 (m, 1H), 4.42 (dd, J = 8.4, 3.6 Hz, 1H), 4.37 and 4.32 (d each, J = 8.7 Hz, total 1H), 3.91 (m, 1H), 3.61 (m, 1H), 3.30 (m, 1H), 3.17 (dd, J = 14.7, 8.4 Hz, 1H), 2.25 (m, 1H), 2.01 and 1.83 (m each, total 3H), 1.25 and 1.18 (d each, J = 6.9 Hz, total 3H).

$[\alpha]_D^{23}$ −52.0° (c 1.0, H_2O).

Anal. Calcd for $C_{16}H_{20}N_4O_6S{\cdot}1.5H_2O$: C, 45.38; H, 5.48; N, 13.23; S, 7.57. Found: C, 45.61; H, 5.34; N, 13.28; S, 7.69.

TLC R_f (c) 0.35.

*1-{N-[(4S,5S)-5-Methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2S)-N-pentylpyrrolidine-2-carboxamide (**76**).* To an ice cooled solution of {[(4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-proline (**75**) (0.300 g, 0.757 mmol) and HOSu (0.087 g, 0.757 mmol) in DMF (30.0 mL), DCC (0.170 g, 0.840 mmol) was added and stirred for 1 h. After the ice bath was removed, the reaction mixture was stirred continuously for 3 h. Next, *n*-pentylamine (0.130 g, 1.49 mmol) was added, and the stirring was continued for 16 h. The precipitate was filtered off and the filtrate was concentrated under reduced pressure. The residue was purified by gel-filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H_2O/MeOH). The purified fractions were concentrated in vacuo, and the residue was lyophilized to afford the title compound **76** (0.230 g, 65%) as a white amorphous powder.

IR (KBr): 3292, 3084, 1753, 1646, 1542, 1445, 1234, 1098 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ 8.97 and 8.95 (d each, J = 2.1 Hz, total 1H), 7.44 and 7.35 (d each, J = 2.1 Hz, total 1H), 5.00 (t, J = 6.9 Hz, 1H), 4.91 (m, 1H), 4.37 (dd, J = 10.5, 4.2 Hz, 1H), 4.35 and 4.33 (d each, J = 9.0 Hz, total 1H), 3.87 (m, 1H), 3.60−3.30 (m, 5H), 2.30−1.70 (m, 4H), 1.51 (m, 2H), 1.31 (m, 4H), 1.25 and 1.20 (d each, J = 6.6 Hz, total 3H), 0.90 (t, J = 6.9 Hz, 3H).

$[\alpha]_D^{25}$ −53.9° (c 1.0, MeOH).

Anal. Calcd for $C_{21}H_{31}N_5O_5S{\cdot}H_2O$: C, 52.16; H, 6.88; N, 14.48; S, 6.63. Found: C, 51.92; H, 6.88; N, 14.73; S, 6.48.

TLC R_f (b) 0.53.

*1-{N-[(4S,5S)-5-Methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2S)-N-(tert-butyl)pyrrolidine-2-carboxamide (**77**).* To an ice cooled solution of {[(4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-L-proline (**75**) (0.300 g, 0.757 mmol) and HOSu (0.087 g, 0.757 mmol) in DMF (30.0 mL), DCC (0.170 g, 0.840 mmol) was added and stirred for 1 h. After the ice bath was removed and the reaction mixture was stirred continuously for 4 h, *tert*-butylamine (0.110 g, 1.52 mmol) was added and stirred for 16 h. The precipitate was filtered off and the filtrate was concentrated under reduced pressure. The residue was purified by gel-filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H_2O/MeOH) and by silica gel column chromatography (eluent: $CHCl_3$/MeOH). The purified fractions were concentrated in vacuo, and the residue was lyophilized to afford the title compound **77** (0.210 g, 62%) as a white amorphous powder.

IR (KBr): 3326, 1753, 1647, 1542, 1449, 1227, 1096 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ 8.97 and 8.95 (d each, J = 1.8 Hz, total 1H), 7.42 and 7.34 (d each, J = 1.8 Hz, total 1H), 5.06 (dd, J = 8.1, 5.4 Hz, 1H), 4.90 (m, 1H), 4.34 (t, J = 8.7 Hz, 1H), 4.31 (d, J = 8.7 Hz, 1H), 3.90−3.60 (m, 2H), 3.37 (dd, J = 15.3, 5.4 Hz, 1H), 3.19 (dd, J = 15.3, 8.1 Hz, 1H), 2.30−1.70 (m, 4H), 1.33 (s, 9H), 1.25 and 1.18 (d each, J = 6.3 Hz, total 3H).

$[\alpha]_D^{25}$ −55.3° (c 1.0, MeOH).

Anal. Calcd for $C_{20}H_{29}N_5O_5S{\cdot}1.5H_2O$: C, 50.20; H, 6.74; N, 14.63; S, 6.70. Found: C, 50.21; H, 6.68; N, 14.78; S, 6.85.

TLC R_f (b) 0.53.

*{[(4R,5S)-5-Methyl-2-oxooxazolidine-4-ylmethyl]-3-(thiazol-4-yl)-L-alanyl}-L-prolinamide (**78**).* To an ice cooled solution of [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**45**) (0.556 g, 1.09 mmol) in DMF (5.00 mL), triethylamine (0.560 mL, 4.02 mmol) and (4*R*,5*S*)-5-methyl-2-oxooxazolidin-4-ylmethyl trifluoromethanesulfonate (**3k**) (0.262 g, 0.995 mmol) were added under stirring for 10 min. After the ice bath was removed and the reaction mixture was stirred continuously for 10 h, the precipitate was filtered off, and the filtrate was concentrated under reduced pressure.

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The residue was purified by gel-filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H_2O/MeOH). The purified fractions were concentrated in vacuo, and the residue was lyophilized to afford the title compound **78** (0.086 g, 27%) as a white amorphous powder.

IR (KBr): 3397, 2979, 2949, 1742, 1678, 1631, 1515, 1437, 1325, 1245, 1079 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.98 and 8.93 (d each, J = 2.1 Hz, total 1H), 7.39 and 7.29 (d each, J = 2.1 Hz, total 1H), 4.86−4.68 (m, 1H), 4.46 (dd, J = 8.7, 3.6 Hz, 1H), 4.13 and 3.86 (t each, J = 5.4 Hz, total 1H), 3.82−3.70 (m, 1H), 3.62 and 3.38 (m each, total 2H), 3.15 and 3.02 (m each, total 2H), 2.80−2.70 (m, 1H), 2.49 and 2.40 (dd each, J = 12.3, 7.5 Hz, total 1H), 2.25−1.70 (m, 4H), 1.28 and 1.26 (d each, J = 6.3 Hz, total 3H).

$[\alpha]_D^{24}$ −10.1° (c 0.50, H_2O).

Anal. Calcd for $C_{16}H_{23}N_5O_4S{\cdot}0.6H_2O$: C, 48.99; H, 6.22; N, 17.85; S, 8.17. Found: C, 48.98; H, 6.12; N, 17.81; S, 6.26.

TLC R_f (b) 0.36.

*1-{N-[(4S,5S)-(5-Methyl-2-oxooxazolidine-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2R)-2-methylpyrrolidine trihydrate (**79**).* 1-{*N*-[(4*S*,5*S*)-(5-Methyl-2-oxooxazolidine-4-yl)-carbonyl]-3-(thiazol-4-yl)-L-alanyl}-(2*R*)-2-methylpyrrolidine (**70**) (5.00 g, 13.6 mmol) was dissolved in hot water (120 mL). After the solution was cooled to room temperature, seed crystals were added and concentrated under reduced pressure until the total volume was about one-sixth volume. The resultant slurry was filtered and washed with cold water to give the title compound **79** (5.34 g, 93%) as colorless crystals.

mp 194−196 °C.

IR (Nujol): 3498, 3276, 1754, 1682, 1609, 1550, 1464, 1378, 1235, 1089 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ 8.97 and 8.96 (d each, J = 2.1 Hz, total 1H), 7.34 and 7.33 (d each, J = 2.1 Hz, total 1H), 5.19 and 5.04 (t each, J = 7.5 Hz, total 1H), 4.92 (dq, J = 8.7, 6.6 Hz, 1H), 4.36 and 4.35 (d each, J = 8.7 Hz, total 1H), 4.07 and 3.92 (m each, total 1H), 3.78 (m, 1H), 3.42 (m, 1H), 3.22 (m, 2H), 2.00−1.50 (m, 4H), 1.28 and 1.22 (d each, J = 6.6 Hz, total 3H), 1.21 and 1.02 (d each, J = 6.6 Hz, total 3H).

^{13}C NMR (75 MHz, CD_3OD): δ 170.47, 1760.33, 170.16, 169.83, 161.30, 155.04, 154.94, 152.97, 152.92, 117.33, 76.12, 76.05, 59.40, 59.33, 54.43, 54.17, 51.94, 51.82, 47.63, 46.11, 34.63, 33.54, 33.26, 32.20, 24.37, 21.86, 20.61, 18.87, 15.83.

$[\alpha]_D^{25}$ −1.9° (c 1.0, H_2O).

Anal. Calcd for $C_{16}H_{22}N_4O_4S{\cdot}3H_2O$: C, 45.70; H, 6.71; N, 13.33; S, 7.63; H_2O, 12.9. Found: C, 45.52; H, 6.49; N, 13.46; S, 7.59; H_2O, 12.8 (K.F.).

TLC R_f (b) 0.56.

Chiral HPLC analysis was performed by using an isocratic solvent system of 0.05 M aqueous phosphate buffer (pH 6.0)/CH_3CN (80/20) using a CAPCELLPACK (Shiseido) C-18 column with a flow rate of 1 mL/min.

t_R = 8.07 min.

Experiments Using Laboratory Animals. All experiments using laboratory animals were conducted in accordance with the guideline by the Animal Care and Use Committee in Shionogi.

Anti Reserpine-Induced Hypothermia Effect in Mice. Male ddY mice were purchased from SLC Japan Inc. at the age of 6 weeks. After quarantine for 1 week, the mice were placed in animal compartments with a controlled room temperature of approximately 25 °C and relative humidity of approximately 60%, and a light cycle time of 12 h [light (8:00−20:00)/dark (20:00−8:00)]. Reserpine-induced hypothermia was conducted by the following method.[14] The mice used had rectal temperatures of 30 °C or lower about 18 h after subcutaneous administration of reserpine (3 mg/kg, 1 mg/mL reserpine injection; Daiichi, Tokyo, Japan). TRH and TRH mimetics were dissolved in saline. Rectal temperature was measured with a thermistor (MGA-III, Nihon Kohden) before and after oral administration of TRH and TRH mimetics up to 7 h after a dose of 50 and 5 μmol/kg/mL, respectively. The antagonistic effect of the test drugs on reserpine-induced hypothermia was evaluated on the basis of the area under the temperature−time curve after dosing (AUC_{0-7h}). All of the mice used in the experiments were killed immediately after the last measurement.

TRH Receptor Binding. Male Sprague-Dawley rats (300−400 g body weight) were sacrificed and dissected on ice to obtain the whole brain excluding the olfactory bulb. The brain was homogenized with glass/Teflon homogenizer (20 strokes) in 20-fold volume of 20 mM phosphate buffer (pH 7.4) and centrifuged at 40 000g for 30 min. The pellet was washed and re-centrifuged under the same conditions. The obtained pellet was suspended in 50-fold volume of 20 mM phosphate buffer (pH 7.4). The TRH receptor binding was conducted with [^{3}H]-(3-Me-His^2)-TRH (Daiichi Chemical, specific activity 82.5 Ci/mmol) and the crude membrane preparation (ca. 0.4 mg protein). The tracer, test compounds, and the membrane preparation were incubated in 0.2 mL of phosphate buffer (20 mM, pH 7.4) in ice cold water for 2 h. After the incubation, the sample was filtered through a Whatman GF/C filter, and the filter was washed three times with the buffer, and then the retained radioactivity was counted with a liquid scintillation counter. The nonspecific binding was measured in the presence of 10 μM TRH (BACHEM). The saturation and inhibition binding studies were conducted with 0.125−8 and 2 nM of [^{3}H]-(3-Me-His^2)-TRH. The K_i value was calculated with the following formula: $K_i = IC_{50}/1 + (\text{ligand}/K_d)$.

PK Experiments of TRH Mimetics 64, 68−70 in Rat. Male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. at the age of 7 weeks. After quarantine for 1 week in the Animal Care Laboratory (Shionogi & Co., Ltd.), the rats were placed in animal compartments with a controlled room temperature of approximately 25 °C and relative humidity of approximately 60%, and a light cycle time of 12 h [light (8:00−20:00)/dark (20:00−8:00)]. All animal studies were conducted with the approval of the Institutional Animal Care and Use Committees of Shionogi Research Laboratories (n = 2, 302−364 g body weight). For oral administration, 2 μmol portions of TRH and TRH mimetics mixture as cassette dosing[61,62] were accurately weighed and dissolved in the dimethyl sulfoxide/0.5% methyl cellulose aq. sol. = 1/4 to obtain the dosing solution with the target concentration and dose of 2 μmol/5 mL/kg. For intravenous administration, 1 μmol portions of TRH and TRH mimetics mixture as cassette dosing were accurately weighed and dissolved in the dimethyl sulfoxide/propylene glycol = 1/1 to obtain the dosing solution with the target concentration and dose of 1 μmol/1 mL/kg. Blood (approximately 0.3 mL) was collected from the jugular vein directory using a heparinized syringe at the scheduled time after administration. The samples were immediately centrifuged in approximately 10 000g for 5 min at 4 °C to obtain plasma, which was transferred into a tube and stored in a freezer at approximately −20 °C until the determination of the concentrations of TRH and TRH

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mimetics. Methanol/acetonitrile = 1/1 mixture was added to the plasma with mixing in a tube with a mixer and centrifugation in approximately 10 000*g* for 5 min at 4 °C to obtain the supernatant. The supernatant was injected into LC/MS/MS systems (ESI positive mode), which was a Qtrap 5500 system (AB SCIEX Pte. Ltd.) to determine the plasma concentration of TRH and TRH mimetics.

Ara-C-Induced Cerebellar Ataxia in Rat. Male and female neonates from Sprague-Dawley pregnant rats (purchased from Clea Japan at 13−17 days of pregnancy) were used. Cytosine arabinoside (Ara-C) was prepared using physiological saline before use and was subcutaneously injected at 60 mg/kg into the dorsal region at age of 2 days and age of 3 days (the day of confirming delivery: age of 0 day). Ara-C-administered animals were housed in room temperature: 22−27 °C, relative humidity: 25−74%, lighting period: 12 h from 7:00. Animals were allowed free access to diets (Clea Japan, CA-1) and water (tap water) by the experiment at age of 3−5 weeks. On the measurement of ataxia, each animal was placed on the center of the circular open field (75 cm in diameter, 25 parts). The number of crossing parts (number of ambulation) and the number of falls for 3 min were measured to calculate fall index ([number of falls]/[number of ambulation]). On the basis of the fall index, the severity of ataxia was assessed. To equalize the severity of ataxia among animals, the fall index ([number of falls]/[number of ambulation]) was measured at baseline (in the morning on the day of experiment for the day before initiation of the experiment for the 7 day repeated-dose experiment) for each animal except animals that did not fall down. Animals with the baseline fall index ranging 1.71−2.19 were included in the study. The severity of ataxia was assessed at 24 h after final administration in the 7 days of repeated oral dose. Animals in the control group were administered physiological saline.

TSH Releasing Activity in Rat. Animal rearing conditions: male Sprague-Dawley rats were purchased from Nippon Clea Co. Ltd at the age of 7 weeks. After quarantine for 1 week in the Animal Care Laboratory (Shionogi & Co., Ltd.), the rats were placed in animal compartments with a controlled room temperature of approximately 25 °C and relative humidity of approximately 60%, and a light cycle time of 12 h [light (7:00−19:00)/dark (19:00−7:00)]. At least n = 4, 258−320 g body weight. For oral administration, 2 mg portions of TRH mimetics were accurately weighed and dissolved in the water to obtain the dosing solution with the target concentration and dose of 0.1 and 1 mg/2 mL/kg. Blood (approximately 3 mL) was collected by decapitation at the scheduled time after oral administration to fed rats. The blood samples were placed for 30 min at room temperature (25 °C) and centrifuged in approximately 10 000*g* for 5 min at 4 °C using a centrifuge to obtain serum, which was transferred into a tube and stored in a freezer at approximately −20 °C until determination of the concentrations of TSH levels. Serum TSH levels were determined by the assay kit which was BIOTRAKTM Rat thyroid stimulating hormone [125I] assay system with magnetic separation, Amersham Pharmacia Biotech, code: RPA554.

PK Experiments of 79 in Rat. Male Wistar rats were purchased from SLC Japan Inc. at the age of 10 or 11 weeks. After quarantine for 1 week in the Animal Care Laboratory (Shionogi & Co., Ltd.), the rats were placed in animal compartments with a controlled room temperature of approximately 25 °C and relative humidity of approximately 60%, and a light cycle time of 12 h [light (8:00−20:00)/dark (20:00−8:00)]. In the fasting study, the rats were reared under fasting condition from the evening of the day before administration until the 12 h-sampling after administration. Each rat was subjected to surgery to insert a cannula tube into the jugular vein under isoflurane anesthesia at 2 or 3 days before administration. At least n = 4, 230−274 g body weight. For oral administration, 40 mg portions of TRH and TRH mimetics were accurately weighed and dissolved in the saline to obtain the dosing solution with the target concentration and dose of 40 mg/4 mL/kg. For intravenous administration, 8 mg portions of TRH and TRH mimetics were accurately weighed and dissolved in the saline to obtain the dosing solution with the target concentration and dose of 8 mg/1.6 mL/kg. Blood (approximately 0.3 mL) was collected from the cannula inserted into the jugular vein using a heparinized syringe at the scheduled time after administration. The samples were immediately centrifuged in approximately 10 000*g* for 5 min at 4 °C using a centrifuge to obtain plasma, which was transferred into a tube and stored in a freezer at approximately −20 °C until determination of the concentrations of TRH and TRH mimetics. Methanol was added to the plasma with mixing in a tube with a mixer and centrifugation at approximately 10 000*g* for 5 min at 4 °C to obtain the supernatant. The supernatant was injected into HPLC systems, which was Waters 805 system (Waters 717 auto sampler, Waters 486 Tunable Absorbance detector, Waters 600 Controller) to determine the plasma concentration of TRH and TRH mimetics.

Brain Kp Experiments of 79 in Rat. Male Sprague-Dawley rats were purchased from Nippon Clea Co. Ltd at the age of 7 weeks. After quarantine for 1 week in the Animal Care Laboratory (Shionogi & Co., Ltd.), the rats were placed in animal compartments with a controlled room temperature of approximately 23 °C and relative humidity of approximately 55%, and a light cycle time of 12 h [light (8:00−20:00)/dark (20:00−8:00)]. In the fasting study, the rats were reared under fasting condition from the evening of the day before administration until the 12 h-sampling after administration. n = 4, 262−312 g body weight. Each rat was subjected to surgery to insert a cannula tube into the jugular vein and micro dialysis cannula tube into ventral tegmental area of brain under pentobarbital anesthesia at 3 days before administration. For oral administration, 40 mg portions of **79** was accurately weighed and dissolved in the saline to obtain the dosing solution with the target concentration and dose of 40 mg/2 mL/kg. Blood (approximately 0.3 mL) was collected from the cannula inserted into the jugular vein using a heparinized syringe at the scheduled time after administration. The samples were immediately centrifuged in approximately 3000*g* for 5 min at 4 °C using a centrifuge to obtain plasma, which was transferred into a tube and stored in a freezer at approximately −20 °C until determination of the concentrations of **79**. Methanol was added to the plasma with mixing in a tube with a mixer and centrifugation at 10 000*g* for 5 min at 4 °C to obtain the supernatant. Micro dialysis solution was collected from the cannula inserted into the ventral tegmental area of brain using a micro syringe pump (1 μL/min of Ringer solution) at the scheduled time after administration.

The supernatant and dialysis solution were injected into HPLC systems with a mass spectrometer, which was Waters 606 system (Waters 717 auto sampler, Thermo Quest

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TSQ7000 mass spectrometer) to determine the plasma and brain dialysis concentration of **79**.

Stability Study in Rat Plasma and Brain Homogenate. Animal rearing conditions: male Sprague-Dawley rats were purchased from Nippon Clea Co. Ltd at the age of 7 weeks. After quarantine for 1 week in the Animal Care Laboratory (Shionogi & Co., Ltd.), the rats were placed in animal compartments with a controlled room temperature of approximately 25 °C and relative humidity of approximately 60%, and a light cycle time of 12 h [light (8:00−20:00)/dark (20:00−8:00)]. **79** in vitro stability test in plasma and brain homogenate (n = 5, 292−306 g body weight).

Test concentration: approximately 1 μg/mL. Nonfasted rats were anesthetized under ether and exsanguinated from the abdominal aorta. The blood was centrifuged (3000g, 5 min) and the plasma collected. Brains were then promptly extracted, divided into cerebrum and cerebellum, nine volumes of phosphate buffered saline (PBS) solution (Dulbecco's PBS (-): Nissui Co.) added, and 10% homogenates prepared in a Polytron homogenizer. Five milliliter quantities of the plasma, cerebrum, and cerebellum homogenates (5 samples of each) were placed in 50 mL capacity tubes, cooled in ice, and mixed with 50 μL of each drug sample solution adjusted to approximately 0.1 mg/kg. Sampling (0 min) was then promptly carried out. Each test solution was left on a water bath at 37 °C, and sampling of 0.1 mL quantities was carried out after 5, 15, 30, 60, 120, and 180 min. Three volumes of methanol was added and mixed with each sample of test solution, the mixture was centrifuged, and the supernatant was collected and stored at -20 °C until determined by TLC. The supernatants were spotted onto TLC plates (20 × 20 cm silica gel 60 F254, Merck Co.) and promptly developed with the following mixed solvents: $CHCl_3$/MeOH/H_2O = 32/6/0.5. After TLC development, [^{14}C]-**79** was kept in contact with X-ray film for 2−3 weeks, and the positions of the labeled entities was confirmed after development. Unchanged drug and other metabolites were separately scraped off the plates and put into glass vials. Distilled water (1 mL) and 15 mL of Pico-Fluor 40 were added and left overnight, and then the radioactivity in each sample was measured by liquid scintillation counter.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01481.

> Synthetic procedure and analytical data for the N-terminus part fragments **3a−h**, the middle-part fragments **4a−b**, **7−9**, **12−17**, the C-terminus part fragments **21−33**, the *N*-Boc and the *N*-Cbz dipeptide mimetics **34−44**, and the dipeptide mimetics **45−55** and X-ray crystallographic data of **79** (PDF)

Accession Codes

Crystal structure coordinates and structure factors for **79** have been deposited to the CCDC with the accession number 1442496.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TRH, thyrotropin-releasing hormone;; CNS, central nervous system; SCD, spinocerebellar degeneration; TSH, thyrotropin-stimulating hormone; Me, methyl; Bn, benzyl; ^{t}Bu, *tert*-butyl; MeOH, methanol; EtOH, ethanol; BnOH, benzyl alcohol; Et_2O, diethyl ether; MEK, methyl ethyl ketone; Et_3N, triethylamine; TsOH·H_2O, *p*-toluenesulfonic acid monohydrate; Tf_2O, trifluoromethanesulfonic anhydride; Tf, trifluoromethanesulfonyl; Ac_2O, acetic anhydride; DMAP, 4-dimethylaminopyridine; Boc_2O, di-*tert*-butyl dicarbonate; MeI, iodomethane; BnBr, benzyl bromide; HOBt, *N*-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; TLC, thin-layer chromatography; t_R, retention time; K.F., Karl Fischer

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