Regular Article

Synthesis and Evaluation of *in Vivo* Anti-hypothermic Effect of the N- and C-Terminus Modified Thyrotropin-Releasing Hormone Mimetic: [(4*S*,5*S*)-(5-Methyl-2-oxooxazolidine-4-yl)-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide

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We explored orally effective thyrotropin-releasing hormone (TRH) mimetics, which show high central nervous system effects in structure–activity relationship studies based on *in vivo* antagonistic activity on reserpine-induced hypothermia (anti-hypothermic effect) in mice starting from TRH. This led us to the TRH mimetic: [(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide **1**, which shows a higher anti-hypothermic effect compared with that of TRH after oral administration. We next attempted further chemical modification of the N- and C-terminus of **1** to find more orally effective TRH mimetics. As a result, we obtained several N- and C-terminus modified TRH mimetics which showed high anti-hypothermic effects.

Key words thyrotropin-releasing hormone (TRH); TRH mimetic; lipophilicity; oral administration; anti-hypothermic effect; TRH-glycine

Introduction

Thyrotropin-releasing hormone (TRH) is a hypothalamus hormone isolated from pigs or sheep for the first time. The chemical structure of TRH revealed it to be L-pyrroglutamyl-L-histidyl-L-prolinamide **2**^{1,2)} (Fig. 1). TRH is a neuropeptide distributed in high concentration in the brain and has hormonal effects such as acceleration of the synthesis and secretion of thyroid stimulating hormone (TSH) from the anterior pituitary.^{3,4)} TRH also has central nervous system (CNS) effects such as neuromodulation.^{5,6)} These CNS effects of TRH have been applied to the treatment of CNS disorders.^{7–33)} For example, TRH tartrate^{7–9)} and taltirelin hydrate **3**^{10–13)} have been used for the treatment of spinocerebellar degeneration (SCD), with taltirelin hydrate being launched in Japan as an orally active agent (Fig. 1). In other case, posatirelin **4**^{14,15)} or JTP-2942 **5**^{16–18)} have been developed in clinical trials for vascular dementia or Alzheimer's disease (Fig. 1). Recently, we have reported on the novel TRH mimetic: [(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide **1** (Fig. 1), which was found after the structure–activity relationship (SAR) study based on *clogP* value in order to penetrate the biological membranes and show the CNS effect. It shows 70-fold higher *in vivo* antagonistic activity on reserpine-induced hypothermia (anti-hypothermic effect) in mice than that of TRH after oral administration.³⁴⁾ We considered that increasing the lipophilicity of **1** (*clogP* = −0.53) compared with TRH (*clogP* = −2.8) might lead to pass through the biological membrane such as intestine, blood–brain barrier (BBB) and show higher anti-hypothermic effect.

On the other hand, TRH is biosynthesized from a TRH precursor, TRH-glycine (TRH-Gly) **6**, a tetrapeptide which is degraded by a specific enzyme in the brain and displays several biological activities^{35–37)} (Fig. 1). The biological activities of **6** express *via* TRH receptor (TRH-R), however, its IC₅₀ for TRH-R is much lower (IC₅₀ = 12 μM) than that of TRH (IC₅₀ = 25 nM).³⁷⁾ Moreover, tetra- or pentapeptide type analogues also have CNS effects.³⁸⁾ Tetrapeptide type analogue **7** shows antidepressant activity,³⁹⁾ and pentapeptide type analogue JAK4D **8** binds to a distinct TRH-R subtype and is effective in several neurodegenerative models^{40,41)} (Fig. 1).

Although many TRH mimetics were reported, the biologically active N-modified type of pyrroglutamate residue of TRH or tetrapeptide type mimetics such as **6** were not reported.

Here, we report the chemical modification of the N- and C-terminus of **1** to increase its lipophilicity and the brain permeability. A SAR study conducted mainly on the *in vivo* anti-hypothermic effect after oral administration. The N- and C-terminus modifications were done at the 3-position of the oxooxazolidine ring (**X**) and the 2-position of pyrrolidine (**Y**). Also, we tried to introduce a glycine moiety to the C-terminus of **1** based on the findings described above (Fig. 2).

Results and Discussion

Chemistry The N-terminus modified TRH mimetics **9–14** and **28–32** were synthesized by methods 1 and 2, respectively.

Method 1) The synthetic route of the N-terminus modified TRH mimetics **9–14** is shown in Chart 1. Chemical modification at the N-terminus was accomplished directly from **1**. The

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Fig. 1. Chemical Structure of TRH Mimetic **1**, TRH **2**, TRH Analogues (Taltirelin Hydrate **3**, Positirelin **4** and JTP-2942 **5**), TRH-Gly **6**, Tetrapeptide Type Analogue **7** and Pentapeptide Type Analogue **8**

Fig. 2. Chemical Modification of the N- and C-Terminus around **1**

Reagents and conditions: (a) aq. HCHO, EtOH, room temperature (r.t.); (b) Ac₂O, r.t., 74%; (c) Ac₂O, pyridine, r.t., 68%; (d) aq. HCHO, amines (piperidine, morpholine or 4-methylpiperazine), EtOH, r.t., 53–69%.

Chart 1. Synthetic Method of the N-Terminus Modified TRH Mimetics **9–14**

Reagents and conditions: (a) BnOH, DMAP, DCC, THF, r.t., 55%; (b) alkyl halide or acyl halide, *tert*-BuOK or NaH, THF or *N,N*-dimethylformamide (DMF), 0°C to r.t., 24–92%; (c) H₂, Pd/C, MeOH, r.t., 55%–quant; (d) 27, HOSu, triethylamine (Et₃N), DCC, DMF, 0°C to r.t., 30–56%.

Chart 2. Synthetic Method of the N-Terminus Modified TRH Mimetics 28–32

Reagents and conditions: (a) IPA or BnOH, HOBT, DCC, DMAP, DMF, 0°C to r.t., 89–95%; (b) 34 or 35, HOBT, DCC, Et₃N, DMF, 0°C to r.t., 78–89%; (c) HCl in ethyl acetate, r.t., 95%–quant.

Chart 3. Synthetic Method of the C-Terminus Fragment 40–43

Reagents and conditions: (a) 40–43, HOBT, DCC, DMF, 0°C to r.t., 72–94%; (b) aq. LiOH, THF–H₂O, 0°C to r.t., 52–94%; (c) HCl in ethyl acetate, r.t., 74%–quant; (d) 15, HOSu, DCC, DMF, 0°C to r.t., 41–74%; (e) 60, HOSu, DCC, Et₃N, DMF, 0°C to r.t., 70%.

Chart 4. Synthetic Method of the C-Terminus Modified TRH Mimetics 54–59

3-position of the oxooxazolidine ring of **1** was alkylated with formaldehyde to afford hydroxymethyl derivative **9** and then treated with acetic anhydride (Ac_2O) to give acetoxymethyl derivative **10**. The 3-position of the oxooxazolidine ring of **1** was also acetylated directly with Ac_2O to afford the acetyl (Ac) derivative **11**. Finally, Mannich base type modification of **1** was accomplished with three kinds of amine (piperidine, morpholine and 4-methylpiperazine) and formaldehyde to afford TRH mimetic **12–14**, respectively.

Method 2) The synthetic route of the N-terminus modified TRH mimetics **28–32** is shown in Chart 2. The (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid **15**^{42,43} was esterified with benzyl alcohol (BnOH) in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to afford benzyl ester derivative **16**. The 3-position of the oxooxazolidine ring of **16** was alkylated or acylated with several alkyl halide or acyl chloride using potassium *tert*-butoxide or sodium hydride as bases to afford 3-position modified oxooxazolidine derivatives **17–21**, respectively. The benzyl groups of **17–21** were deprotected by catalytic hydrogenation to give carboxylate derivatives **22–26**, respectively. Compounds **22–26** were coupled with [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride **27**³⁴ in the presence of *N*-hydroxysuccinimide (HOSu) and DCC to afford TRH mimetics **28–32**, respectively. The detailed synthetic method, yields and physical data of intermediates **16–26** are given in the Supplementary Materials.

The C-terminus modified TRH mimetics **54–59** were synthesized by the fragment synthesis and the peptide synthesis shown in Charts 3 and 4, respectively.

The C-terminus fragments **40–43** were synthesized from the readily available *N*-*tert*-butoxycarbonyl (Boc)-L-proline **33** (Chart 3). *N*-Boc-L-proline **33** was coupled with isopropyl

alcohol (IPA), BnOH, isopropyl glycinate *p*-toluenesulfonate **34** or benzyl glycinate *p*-toluenesulfonate **35** in the presence of DCC, *N*-hydroxybenzotriazole (HOBt) and DMAP to afford ester derivatives **36–39**, respectively. The Boc groups of **36–39** were deprotected with hydrogen chloride in ethyl acetate solution to give the C-terminus fragments **40–43**, respectively. The detailed synthetic method, yields and physical data of intermediates **34–43** are presented in the Supplementary Materials.

TRH mimetics **54–59** were synthesized based on our previous method³⁴ (Chart 4). The C-terminus fragments **40–43** were coupled with *N*-Boc-3-(thiazol-4-yl)-L-alanine **44**^{44,45} to afford *N*-Boc dipeptide **45**, **46** and tripeptide **48**, **49**, respectively. The benzyl group of benzyl ester derivative **46** was deprotected with aqueous lithium hydroxide solution to afford carboxylate **47**. The Boc groups of **45**, **47–49** were deprotected under acidic condition to give dipeptide **50**, **51** and tripeptide **52**, **53**, respectively. Di- or tripeptide **50–53** were coupled with carboxylate **15** in a similar manner to that described in Chart 1 to give TRH mimetics **54**, **55** and tetrapeptide type TRH mimetics **57**, **58**, respectively. TRH mimetic **55** was coupled with *tert*-butyl glycinate hydrochloride **60** to afford tetrapeptide type **56**. The benzyl group of **58** was deprotected with aqueous lithium hydroxide solution to give tetrapeptide type **59**. The detailed synthetic method, yields and physical data of intermediates **45–53** are shown in the Supplementary Materials.

Physicochemical Properties and Anti-hypothermic Effects of TRH Mimetics The physicochemical properties were estimated by $c\log P$, which is the log of the octanol/water partition coefficient, calculated using ChemBioDraw Ultra. TRH and TRH analogues promote the secretion of catecholamine against reserpine in the brain and increase body

Table 1. Physicochemical Properties and Anti-hypothermic Effects of TRH Mimetics

Compounds	X	Y	$c\log P^{a)}$	Dose ^{b)} ($\mu\text{mol/kg}$)	$\Delta AUC_{0-7h}^{c)}$ ($^{\circ}\text{C}\cdot\text{h}$)
TRH	—	—	−2.8	50	9 ± 1
1	H	CONH_2	−0.53	5.0	61 ± 1
10	CH_2OAc	CONH_2	0.32	10	44 ± 2
11	Ac	CONH_2	0.16	10	23 ± 6
13	CH_2 -Morpholino	CONH_2	0.55	10	36 ± 5
28	CH_2COEt	CONH_2	0.59	10	19 ± 1
29	CH_2CO -Morpholino	CONH_2	0.02	10	44 ± 4
30	$\text{CH}_2\text{OCO}^t\text{Bu}$	CONH_2	1.6	10	26 ± 2
31	CO_2Et	CONH_2	0.88	10	11 ± 1
32	CO -Morpholino	CONH_2	0.71	10	-4 ± 1
54	H	CO_2^iPr	1.4	10	25 ± 3
55	H	CO_2H	0.33	10	18 ± 3
56	H	$\text{CONHCH}_2\text{CO}_2^t\text{Bu}$	1.3	10	29 ± 3
57	H	$\text{CONHCH}_2\text{CO}_2^i\text{Pr}$	0.87	10	50 ± 1
59	H	$\text{CONHCH}_2\text{CO}_2\text{H}$	−0.09	10	29 ± 7

a) $c\log P$ was calculated using ChemBioDraw Ultra 14.0. b) Administration doses of TRH, **1** or TRH mimetics were 50, 5.0 or $10\mu\text{mol/kg}$, respectively. c) Each value represents the mean $\pm$ standard deviation (S.D.) of at least three animals. Anti-hypothermic effect for increasing rectal temperature by 1°C per hour in reserpine-induced hypothermia mice. The formula for computation is as follows: AUC_{0-7h} ($^{\circ}\text{C}\cdot\text{h}$): area under the rectal temperature–time curve for 7h. Anti-hypothermic effect (ΔAUC_{0-7h}) = AUC_{0-7h} of compounds $-\Delta AUC_{0-7h}$ of saline.

temperature.²⁵⁾ The anti-hypothermic effects of TRH mimetics **10**, **11**, **13**, **28–32**, **54–57** and **59** were evaluated by their *in vivo* antagonistic activity on reserpine-induced hypothermia in mice.^{10–13)} The mice which were 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 after oral administration of TRH, **1** or TRH mimetics up to 7 h at a dose of 50, 5.0 or 10 μ mol/kg, respectively. The anti-hypothermic effects were measured by the area under the rectal temperature–time curve (AUC_{0-7h}) after dosing. Moreover, the anti-hypothermic effect of each compound was calculated as the ΔAUC_{0-7h} of each compound and saline.^{34,46)} The anti-hypothermic effects (ΔAUC_{0-7h}) of TRH mimetics are shown with the lipophilicity ($c\log P$) and administration doses in Table 1. Rectal temperature–time graphs of TRH mimetics and the detailed calculation method of anti-hypothermic effects are given in the Supplementary Materials (Figure S1–S16).

In the case of the N-terminus modified TRH mimetics, TRH mimetics having alkyl groups such as acetoxymethyl group **10**, 4-morpholinomethyl group **13**, 2-morpholino-2-oxoethyl group **29** showed high anti-hypothermic effect. Presumably, **10** and **13**, which might act as prodrugs of **1**,⁴⁷⁾ were partially converted to **1** during the *in vivo* test and showed high activity. Because acetoxymethyl group of **10** and 4-morpholinomethyl group of **13** might be hydrolyzed and converted to parent drug **1** via unstable amination derivative **9**. Otherwise, TRH mimetics having the 2-oxobutanyl group **28** and the pivaloyloxymethyl group **30** showed low activity. The TRH mimetics having the piperidinomethyl group **12** and the 4-methylpiperazinomethyl group **14** showed lower activity than that of **13** (data not shown). On the other hand, TRH mimetics having alcy groups such as the acetyl group **11**, ethoxycarbonyl group **31** and morpholine-4-carbonyl group **32** showed lower activity than those with alkyl groups. In particular, **32** did not show activity at all. As a result, **29** had the highest activity among the N-terminus modified TRH mimetics.

In the case of the C-terminus modified TRH mimetics, the one with the isopropylloxycarbonyl group **54** showed higher anti-hypothermic effect than that with the hydroxycarbonyl group **55**. Moreover, the tetrapeptide type mimetic having the isopropyl glycinate moiety **57** showed the highest anti-hypothermic effect among **56**, **57** and **59**. Surprisingly, the tetrapeptide type mimetic with the glycine moiety **59** showed a lower anti-hypothermic effect than that of the isopropyl ester derivative **57**. As a result, **57** showed the highest activity among the C-terminus modified TRH mimetics. The lipophilicities ($c\log P$) of TRH mimetics were not related with the anti-hypothermic effects (ΔAUC_{0-7h}). Moreover, all TRH

mimetics showed high water solubilities (> 10 mg/mL), the correlation between $c\log P$ and solubilities were not unclear.

As a result, we discovered new TRH mimetic **29** and tetrapeptide type mimetic **57**, which showed high anti-hypothermic effects from the N- and C-terminus modification. However, we did not find TRH mimetics which showed higher *in vivo* effects than that of **1**.

Binding Affinity for Rat TRH Receptor TRH-R binding of TRH mimetic **29** and tetrapeptide type mimetic **57**, which showed high anti-hypothermic effect, were evaluated using a rat brain TRH-R which included both TRH receptor type 1 (TRH-R1)⁴⁸⁾ and TRH receptor type 2 (TRH-R2).^{49,50)} The human TRH-R is well conserved when compared with that 90.3% homologous to mouse and 89.2% homologous to rat at the DNA level, respectively.⁵¹⁾ However, it was not possible to distinguish whether compounds bound preferentially to TRH-R1 or TRH-R2. The results of the TRH-R binding assay are shown with TRH and **1** in Table 2.

The N-terminus modified TRH mimetic **29** showed 83-fold lower affinity to TRH-R than that of **1**. On the other hand, tetrapeptide type mimetic **57** showed 21-fold lower affinity than that of **1**. As a result, the binding affinity of the N-terminus is higher than that of the C-terminus. It was reported that the NH of pyroglutamate residue and primary amide group of prolinamide residue of TRH are necessary to form hydrogen bonding between the TRH-R and show biological activity.^{52,53)} Similarly, it is important that the 3-position NH of the oxooxazolidine ring in the N-terminus and primary amide group of **1** to bind strongly to the TRH-R. In contrast, the tetrapeptide type mimetic showed a relatively high affinity for the receptor compared with the N-terminus modified type mimetic. As a result, *in vitro* activity was related with *in vivo* activity. In fact, 83-fold gap of the receptor binding affinities between **1** and **29** reflected 17 °C·h gap of the anti-hypothermic effect. And 21-fold gap of the *in vitro* activities reflected 11 °C·h gap of the *in vivo* effects between **1** and **57**. These facts are similar to that the relationship between *in vitro* human TRH-R binding affinities and *in vivo* activities of rovatirelin and taltirelin.⁵⁴⁾

Table 2. Binding Affinities (K_i) of TRH and TRH Mimetics for Rat TRH Receptor

Compounds	K_i (nM) ^{a)}
TRH	25 ^{b)}
1	3.5 ^{b)}
29	292
57	75

a) K_i values were obtained from the inhibition of [³H]-(3-Me-His²)-TRH using a membrane preparation of the rat whole brain. b) K_i values were referred from our previous report.³⁴⁾

Table 3. PK Properties of TRH Mimetics **29**, **57** and **59** in Rats^{a)}

Compounds	AUC (i.v.) (nM·h)	CL (mL/min/kg)	$t_{1/2}$ (h) ^{b)}	Vd_{ss} (L/kg)	F (%) ^{c)}	bKp	fu (%) ^{d)}	Serum $t_{1/2}$ (h) ^{e)}
1	1290 ^{f)}	13.0 ^{f)}	0.2 ^{f)}	0.19 ^{f)}	1.1 ^{f)}	NC ^{g)}	>99 ^{f)}	28 ^{f)}
29	1320	12.6	0.3	0.21	0.60	0.01	97	>75
57	NC ^{g)}	NC ^{g)}	NC ^{g)}	NC ^{g)}	NC ^{g)}	NC ^{g)}	NC ^{g)}	NC ^{g)}
59	NT ^{h)}	NT ^{h)}	NT ^{h)}	NT ^{h)}	NT ^{h)}	NC ^{g)}	NC ^{g)}	5.6

a) Each value represents the mean of two animals. b) $t_{1/2}$ of the plasma concentration of TRH mimetics after i.v. administration to rats were calculated by interpolation for plasma concentration of terminal phase. c) F values of TRH mimetics after oral administration were calculated using the plasma concentration after i.v. and oral administrations. d) fu : free fraction ratio in rat serum (%). e) serum $t_{1/2}$: the half-life times of TRH mimetics in rat serum (h). f) Each values were referred from our previous report.³⁴⁾ g) NC: Not calculated. h) NT: Not tested.

To evaluate the correlation *in vivo* effect and *in vitro* activity, we examined the pharmacokinetic (PK) properties of **29** and **57**.

PK Properties of TRH Mimetics 29, 57 and 59 in Rat
The clearance (*CL*), half-life times ($t_{1/2}$), volume of distribution at steady state ($V_{d_{ss}}$) and brain penetration ratio (bKp) of the three TRH mimetics **29**, **57** and **59** in rat after intravenous (i.v.) administration were evaluated. Also, the bioavailability (*F*) of these two TRH mimetics in rat after oral administration were evaluated. Moreover, free fraction ratio (*fu*) and stability in serum (serum $t_{1/2}$) were evaluated *in vitro* examinations. These PK properties are shown in Table 3. Here, the PK properties of **1** were referred from our previous report.³⁴⁾

The PK properties of **29** were similar to that of **1**. The brain permeability of **29** was slightly increased because of its lipophilicity. On the other hand, the rat serum stability of **29** was much higher than that of **1**. Although the similar *in vivo* effects between **1** and **29**, the binding affinity of **29** was 83-fold lower than **1**. We considered that increasing the serum stability and brain permeability of **29** were lead to the similar *in vivo* effects between **1** and **29**. On the other hand, the PK properties of **57** were not calculated because its stability in serum was significantly low. Presumably, the isopropyl ester group of **57** was rapidly hydrolyzed by carboxyesterase 1 (CES1) and converted to carboxylate **59**.⁵⁵⁾ Consequently, **57** was not detected in plasma and its PK and *fu* values were not calculated. We speculated that **57** was unstable and the *in vivo* effect of **57** was derived from **59**. Then, we additionally examined the PK properties of **59** after **57** was administrated. Surprisingly, the plasma concentration of **59** was too low to be not calculated (data not shown) although its stability in serum was relatively high. Moreover, it was realized that **57** was degraded and converted to tripeptide **55** as one of the metabolites which showed lower activity than **57** and **59**. We considered that their *in vivo* effects might be depended on their individual intestinal absorption, brain penetration and stability profiles.

Conclusion

We synthesized and evaluated TRH mimetics and tetrapeptide type mimetics around **1** as a lead to find orally active compounds. The N-terminus modified TRH mimetics were synthesized by two methods (Charts 1 and 2). The C-terminus modified mimetics including tetrapeptide types were synthesized in two steps (Charts 3 and 4).

TRH mimetics were evaluated by *in vivo* anti-hypothermic effect in mice after oral administration. As a result, we found the N-terminus modified TRH mimetic **29** having the 2-morpholino-2-oxoethyl group and the C-terminus modified TRH mimetic **57** having the isopropyl glycinate moiety showed high anti-hypothermic effect (Table 1). Next, *in vitro* TRH-R binding affinities and PK properties of **29** and **57** were evaluated (Tables 2, 3). TRH mimetic **57** showed 4-fold higher affinity than that of **29**, however, the binding affinity of **57** was 21-fold lower than that of **1** (Table 2). As to the rat PK properties, **29** indicated in similar to that of **1**. On the other hand, the PK properties of **57** did not be calculated because of the degradation in plasma (Table 3). Then, we speculated the carboxylate **59** was produced by hydrolyzed with carboxyesterase in plasma and it showed the *in vivo* effect. As a result of the PK study of **57** and **59**, the plasma concentration of **59**

was not calculated nevertheless the tripeptide **55** was detected as one of the metabolites after administration of **57**. Therefore, we considered that the *in vivo* effect after oral administration could be depended on their individual intestinal absorption, brain penetration and stability profiles.

Thus, we conducted the SAR study around **1**, we found newly some orally effective the N- and C-terminus modified TRH mimetics. In particular, we discovered N- and C-terminus modified TRH mimetic **29** and **57** which showed high *in vivo* effect. We also indicate new chemical modification point in TRH and its mimetics.

Experimental

General Melting points (mp) were measured by Yanagimoto melting point apparatus and uncorrected. ¹H spectra were taken with a Varian VXR-200 or Gemini-200 300 FT-NMR spectrometer using tetramethylsilane as an internal standard. Optical rotations were determined with a Perkin-Elmer 430 polarimeter. Elemental analyses were performed by Shionogi Research Laboratories (Shionogi & Co., Ltd., 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan). For silica gel column chromatography, Kiesel gel 60 (0.063–0.20 mm, Merck) was employed for the purification. MCI GEL CHP-20P (75–150 g, Mitsubishi Chemical Industries) was utilized with aqueous MeOH as an eluent. TLC was carried out with Merck silica gel 603–254 plates with use of the following mainly three solvent systems: (a) CHCl₃/MeOH (9/1), (b) CHCl₃/MeOH/H₂O (32/6/0.5) and (c) CHCl₃/MeOH/H₂O (6/4/1). The spots were detected under UV irradiation at 254 nm and by the use of phosphomolybdic acid in ethanol solution and ninhydrin sprays.

[(4*S*,5*S*)-3-Acetoxymethyl-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (10**)**
To a solution of [(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**1**) (0.198 g, 0.500 mmol) in EtOH (1.00 mL), triethylamine (0.005 mL, 0.036 mmol) and 37% aqueous formaldehyde solution (0.130 mL, 1.60 mmol) were added and the mixture was stirred for 15 h at room temperature. The reaction mixture was concentrated *in vacuo* to give hydroxymethyl derivative **9**. To a solution of hydroxymethyl derivative **9** in pyridine (10.0 mL), acetic anhydride (1.00 mL, 10.6 mmol) was added and the mixture was stirred for 1 h at room temperature. Toluene (20.0 mL) was added to the mixture and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: CHCl₃/MeOH) to afford the title compound **10** (0.173 g, 74%) as a white amorphous powder.

¹H-NMR (300 MHz, CD₃OD) δ : 8.97 and 8.94 (d each, *J* = 1.8 Hz, total 1H), 7.47 and 7.39 (d each, *J* = 1.8 Hz, total 1H), 5.31 and 5.29 (d each, *J* = 11.4 Hz, total 1H), 5.04 (t, *J* = 6.9 Hz, 1H), 5.01 and 4.98 (d each, *J* = 11.4 Hz, total 1H), 4.80 (m, 1H), 4.59 and 4.56 (d each, *J* = 8.7 Hz, total 1H), 4.41 and 4.30 (dd each, *J* = 8.4, 3.9 Hz, total 1H), 3.87 (m, 1H), 3.50 (m, 1H), 3.40 (dd, *J* = 14.1, 6.6 Hz, 1H), 3.22 (dd, *J* = 14.1, 6.6 Hz, 1H), 2.30–1.70 (m, 4H), 2.05 (s, 3H), 1.30 and 1.24 (d each, *J* = 6.6 Hz, total 3H). $[\alpha]_D^{25}$ –74.6° (c 0.50, H₂O). *Anal.* Calcd for C₁₉H₂₅N₅O₇S·1.1H₂O: C, 46.83; H, 5.63; N, 14.37; S, 6.58. Found: C, 46.87; H, 5.57; N, 14.50; S, 6.63.

[(4*S*,5*S*)-3-Acetyl-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (11**)**
To a solution of [(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)-

carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**1**) (0.125 g, 0.316 mmol) in pyridine (5.00 mL), acetic anhydride (1.20 mL, 12.7 mmol) was added and the mixture was stirred for 16 h at room temperature. Toluene (30.0 mL) was added to the mixture and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: CHCl₃/MeOH) to afford the title compound **11** (0.094 g, 68%) as a white solid.

mp 226–228 °C. ¹H-NMR (300 MHz, CD₃OD) δ : 8.94 and 8.93 (d each, J = 1.8 Hz, total 1H), 7.47 and 7.38 (d each, J = 1.8 Hz, total 1H), 4.97 (t, J = 6.9 Hz, 1H), 4.90–4.80 (m, 2H), 4.41 and 4.25 (dd each, J = 8.7, 3.9 Hz, total 1H), 3.85 (m, 1H), 3.44 (m, 1H), 3.39 (dd, J = 15.0, 7.2 Hz, 1H), 3.23 (dd, J = 15.0, 6.9 Hz, 1H), 2.46 (s, 3H), 2.30–1.80 (m, 4H), 1.40–1.20 (m, 3H). $[\alpha]_{\text{D}}^{25}$ –103.0° (c 0.50, H₂O). *Anal.* Calcd for C₁₈H₂₃N₅O₆S: C, 49.42; H, 5.30; N, 16.01; S, 7.33. Found: C, 49.21; H, 5.34; N, 16.21; S, 7.31.

[(4*S*,5*S*)-3-(2-Oxobutanyl)-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (12**)**

To a solution of [(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**1**) (0.395 g, 1.00 mmol) in EtOH (10.0 mL), 37% formaldehyde in aqueous solution (0.240 mL) and piperidine (0.230 mL, 2.33 mmol) were added and the mixture was stirred for 3 h at 60 °C. The mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent: CHCl₃/MeOH). The purified fractions were collected and concentrated *in vacuo*. To the residue, diethyl ether (10 mL) was added and filtered to afford the title compound **12** (0.262 g, 53%) as a white solid.

¹H-NMR (200 MHz, dimethyl sulfoxide (DMSO)-*d*₆) δ : 9.06 and 9.02 (d each, J = 2.0 Hz, total 1H), 8.81 and 8.59 (d each, J = 8.0 Hz, total 1H), 7.43 (d, J = 2.0 Hz, 1H), 7.34 (brs, 1H), 7.16 and 6.90 (brs, 1H), 4.96 (m, 1H), 4.74 (m, 1H), 4.50 and 4.37 (d each, J = 8.2 Hz, total 1H), 4.22 (m, 1H), 3.95 (d, J = 12.8 Hz, 1H), 3.72 (m, 1H), 3.60 (m, 1H), 3.26 (d, J = 12.8 Hz, 1H), 3.20 (dd, J = 14.0, 5.0 Hz, 1H), 3.04 (dd, J = 14.0, 9.8 Hz, 1H), 2.31 (m, 4 H), 2.10–1.60 (m, 4H), 1.40 (m, 6H), 1.16 and 1.09 (d each, J = 6.4 Hz, total 3H). $[\alpha]_{\text{D}}^{23.5}$ –62.3° (c 0.51, H₂O). *Anal.* Calcd for C₂₂H₃₂N₆O₅S·0.6H₂O: C, 52.49; H, 6.65; N, 16.69; S, 6.30. Found: C, 52.44; H, 6.58; N, 16.84; S, 6.37. In a similar manner, TRH mimetics **13** and **14** were prepared.

[(4*S*,5*S*)-3-(2-Morpholinomethyl)-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (13**)**

N-Aminomethylation of [(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**1**) (0.237 g, 0.600 mmol) with 37% formaldehyde in aqueous solution (0.220 mL) and morpholine (0.180 mL, 2.07 mmol) in EtOH (3.60 mL) yielded the title compound **13** (0.195 g, 66%) as a white solid.

¹H-NMR (300 MHz, CD₃OD) δ : 8.98 and 8.96 (d each, J = 1.8 Hz, total 1H), 7.43 and 7.36 (d each, J = 1.8 Hz, total 1H), 5.08 (dd, J = 7.8, 6.0 Hz, 1H), 4.84 (m, 1H), 4.46 (d, J = 8.4 Hz, 1H), 4.42 (dd, J = 8.4, 3.9 Hz, 1H), 4.07 (d, J = 12.6 Hz, 1H), 3.91 (m, 1H), 3.63 (m, 5H), 3.56 (d, J = 12.6 Hz, 1H), 3.41 (dd, J = 14.4, 6.0 Hz, 1H), 3.19 (dd, J = 14.4, 7.8 Hz, 1H), 2.47 (m, 4H), 2.21 (m, 2H), 2.01 (m, 2H), 1.30 and 1.24 (d each, J = 6.9 Hz, total 3H). $[\alpha]_{\text{D}}^{23.5}$ –61.0° (c 0.50, H₂O). *Anal.* Calcd for C₂₁H₃₀N₆O₆S·0.5Et₂O·1.2H₂O: C, 49.93; H, 6.81; N, 15.19; S, 5.80. Found: C, 49.64; H, 6.45; N, 15.58; S, 5.77.

[(4*S*,5*S*)-3-(4-Methylpiperazinomethyl)-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (14**)** *N*-Aminomethylation of [(4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-yl)carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**1**) (0.395 g, 1.00 mmol) with 37% formaldehyde in aqueous solution (0.240 mL) and *N*-methylpiperazine (0.260 mL, 2.34 mmol) in EtOH (10.0 mL) yielded the title compound **14** (0.350 g, 69%) as a white solid.

¹H-NMR (200 MHz, CD₃OD) δ : 8.98 and 8.96 (d each, J = 2.0 Hz, total 1H), 7.43 and 7.36 (d each, J = 2.0 Hz, total 1H), 5.07 (dd, J = 8.2, 6.4 Hz, 1H), 4.80 (m, 1H), 4.44 (d, J = 8.6 Hz, 1H), 4.41 (dd, J = 8.2, 4.0 Hz, 1H), 4.11 and 4.10 (d each, J = 13.0 Hz, total 1H), 3.90 (m, 1H), 3.56 (d, J = 13.0 Hz, 1H), 3.51 (m, 1H), 3.40 (dd, J = 14.4, 6.4 Hz, 1H), 2.60–2.30 (m, 8H), 2.27 and 2.15 (s each, total 3H), 2.20 (m, 2H), 2.01 (m, 4H), 1.29 and 1.24 (d each, J = 6.2 Hz, total 3H). $[\alpha]_{\text{D}}^{23.5}$ –69.1° (c 0.97, H₂O). *Anal.* Calcd for C₂₂H₃₃N₇O₅S·0.6H₂O: C, 50.97; H, 6.65; N, 18.91; S, 6.18. Found: C, 51.21; H, 6.75; N, 18.59; S, 6.25.

General Procedure for the Synthesis of TRH Mimetics 28–32

[(4*S*,5*S*)-3-(2-Oxobutanyl)-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (28**)**

To an ice cooled solution of (4*S*,5*S*)-3-(2-oxobutanyl)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**17**) (0.500 g, 2.32 mmol) and HOSu (0.270 g, 2.32 mmol) in DMF (12.0 mL), DCC (0.530 g, 2.55 mmol) was added and the mixture was stirred for 0.5 h. After the ice bath was removed, the reaction mixture was stirred continuously for 2 h, [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**27**) (0.792 g, 2.32 mmol) and triethylamine (1.29 mL, 9.28 mmol) were added and the mixture was stirred for 10 h. The precipitate was filtrated off and the filtrate was concentrated under reduced pressure. The residue was purified by gel filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H₂O/MeOH) and by silica gel column chromatography (eluent: CHCl₃/MeOH). The purified fractions were collected and lyophilized to afford the title compound **28** (0.380 g, 35%) as a white amorphous powder.

¹H-NMR (300 MHz, CD₃OD) δ : 8.98 and 8.96 (d each, J = 2.1 Hz, total 1H), 7.43 and 7.35 (d each, J = 2.1 Hz, total 1H), 5.02 (dd, J = 7.5, 6.6 Hz, 1H), 4.92 (m, 1H), 4.49 and 4.48 (d each, J = 9.0 Hz, total 1H), 4.40 (dd, J = 8.4, 4.2 Hz, 1H), 4.34 (d, J = 18.6 Hz, 1H), 3.85 (m, 1H), 3.76 (d, J = 18.6 Hz, 1H), 3.51 (m, 1H), 3.38 (dd, J = 14.7, 6.3 Hz, 1H), 3.17 (dd, J = 14.7, 7.8 Hz, 1H), 2.48 (m, 2H), 2.30–1.80 (m, 4H), 1.26 and 1.21 (d each, J = 6.9 Hz, total 3H), 1.06 and 1.05 (t each, J = 7.8 Hz, total 3H). $[\alpha]_{\text{D}}^{26}$ –80.4° (c 1.0, MeOH). *Anal.* Calcd for C₂₀H₂₇N₅O₆S·1.3H₂O: C, 49.13; H, 6.10; N, 14.32; S, 6.56. Found: C, 49.08; H, 6.08; N, 14.42; S, 6.82.

In a similar manner, TRH mimetics **29–32** were prepared.

[(4*S*,5*S*)-5-Methyl-3-(2-morpholino-2-oxoethyl)-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (29**)**

The condensation of (4*S*,5*S*)-5-methyl-3-(2-morpholino-2-oxoethyl)-2-oxooxazolidine-4-carboxylic acid (**18**) (0.732 g, 2.69 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**27**) (0.781 g, 2.29 mmol) yielded the title compound **29** (0.414 g, 35%) as a white amorphous powder.

¹H-NMR (200 MHz, CD₃OD) δ : 8.98 and 8.96 (d each, J = 2.0 Hz, total 1H), 7.46 and 7.37 (d each, J = 2.0 Hz, total

1H), 5.10–4.90 (m, 2H), 4.51 (d, $J=9.2$ Hz, 1H), 4.40 (dd, $J=8.4, 4.0$ Hz, 1H), 4.36 (d, $J=17.2$ Hz, 1H), 3.95–3.80 (m, 1H), 3.86 (d, $J=17.2$ Hz, 1H), 3.75–3.60 (m, 5H), 3.55–3.45 (m, 4H), 3.39 (dd, $J=14.4, 6.0$ Hz, 1H), 3.18 (dd, $J=14.4, 8.0$ Hz, 1H), 2.30–2.10 (m, 1H), 2.05–1.95 (m, 3H), 1.24 and 1.17 (d each, $J=6.4$ Hz, total 3H). $[\alpha]_D^{25} -79.1^\circ$ (c 1.0, H₂O). *Anal.* Calcd for C₂₂H₃₀N₆O₇S·1.7H₂O: C, 47.77; H, 6.09; N, 15.19; S, 5.80. Found: C, 47.60; H, 5.95; N, 15.41; S, 5.84.

[(4*S*,5*S*)-3-Pivaloyloxymethyl-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**30**)

The condensation of (4*S*,5*S*)-3-pivaloyloxymethyl-5-methyl-2-oxooxazolidine-4-carboxylic acid (**19**) (0.311 g, 1.20 mmol) and [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**27**) (0.757 g, 1.32 mmol) yielded the title compound **30** (0.184 g, 30%) as a white solid.

mp 211–213 °C. ¹H-NMR (300 MHz, CD₃OD) δ : 8.97 and 8.94 (d each, $J=2.1$ Hz, total 1H), 7.48 and 7.40 (d each, $J=2.1$ Hz, total 1H), 5.33 and 5.31 (d each, $J=11.1$ Hz, total 1H), 5.03 (t, $J=6.9$ Hz, 1H), 5.01 and 4.96 (d each, $J=11.1$ Hz, total 1H), 4.84 (m, 1H), 4.58 and 4.54 (d each, $J=8.7$ Hz, total 1H), 4.41 and 4.32 (dd, $J=8.1, 3.9$ Hz, 1H), 3.89 (m, 1H), 3.41 (dd, $J=14.7, 6.6$ Hz, 1H), 3.22 (dd, $J=14.7, 7.2$ Hz, 1H), 2.29 (m, 1H), 2.00 (m, 3H), 1.30 and 1.25 (d each, $J=6.6$ Hz, total 3H), 1.21 and 1.16 (s each, total 9H). $[\alpha]_D^{24} -66.3^\circ$ (c 0.51, MeOH). *Anal.* Calcd for C₂₂H₃₁N₅O₇S: C, 51.86; H, 6.13; N, 13.74; S, 6.29. Found: C, 51.59; H, 6.10; N, 13.82; S, 6.35.

[(4*S*,5*S*)-3-Ethoxycarbonyl-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**31**)

To an ice cooled solution of (4*S*,5*S*)-3-ethoxycarbonyl-5-methyl-2-oxooxazolidine-4-carboxylic acid (**20**) (0.236 g, 1.09 mmol) in THF (5.00 mL), oxalyl chloride (0.250 mL, 2.87 mmol) and small amount of DMF were added and the mixture was stirred for 2.5 h. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in THF (3.00 mL) and added to an ice cooled solution of [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**27**) (0.688 g, 1.20 mmol) and triethylamine (0.610 mL, 4.35 mmol) in DMF (9.00 mL). The mixture was stirred continuously for 1 h. After the ice bath was removed, the reaction mixture was stirred continuously for 18 h. The precipitate was filtrated off and the filtrate was concentrated under reduced pressure. The residue was purified by gel filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H₂O/MeOH) and by silica gel column chromatography (eluent: CHCl₃/MeOH). The purified fractions were collected and lyophilized to afford the title compound **31** (0.188 g, 34%) as a white solid.

mp 126–130 °C. ¹H-NMR (300 MHz, CD₃OD) δ : 8.97 and 8.96 (d each, $J=2.1$ Hz, total 1H), 7.45 and 7.38 (d each, $J=2.1$ Hz, total 1H), 5.01 (t, $J=6.9$ Hz, 1H), 4.84 (m, 1H), 4.78 (d, $J=8.4$ Hz, 1H), 4.41 (dd, $J=8.7, 4.2$ Hz, 1H), 4.20 (q, $J=7.2$ Hz, 2H), 3.88 (m, 1H), 3.48 (m, 1H), 3.40 (dd, $J=14.7, 6.9$ Hz, 1H), 3.20 (dd, $J=14.7, 7.2$ Hz, 1H), 2.19 (m, 1H), 1.99 (m, 3H), 1.37 and 1.30 (d each, $J=6.3$ Hz, total 3H), 1.25 and 1.20 (t each, $J=7.2$ Hz, total 3H). $[\alpha]_D^{23} -94.5^\circ$ (c 0.50, H₂O). *Anal.* Calcd for C₁₉H₂₅N₅O₇S·0.4H₂O: C, 48.07; H, 5.48; N, 14.75; S, 6.75. Found: C, 48.13; H, 5.51; N, 14.86; S, 6.85.

[(4*S*,5*S*)-5-Methyl-3-(morpholine-4-carbonyl)-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinamide (**32**)

To an ice cooled solution of (4*S*,5*S*)-5-methyl-3-(morpholine-4-carbonyl)-2-oxooxazolidine-4-carboxylic acid (**21**) (0.600 g,

2.32 mmol) in dichloromethane (6.00 mL), oxalyl chloride (0.314 mL, 3.71 mmol) and small amount of DMF were added and the mixture was stirred for 2.5 h. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in THF (3.00 mL) and added to an ice cooled solution of [3-(thiazol-4-yl)-L-alanyl]-L-prolinamide dihydrochloride (**27**) (0.792 g, 2.32 mmol) and triethylamine (1.29 mL, 9.28 mmol) in DMF (9.00 mL). The mixture was stirred continuously for 10 h. The precipitate was filtrated off and the filtrate was concentrated under reduced pressure. The residue was purified by gel filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H₂O/MeOH). The purified fractions were collected and lyophilized to afford the title compound **32** (0.663 g, 56%) as a white amorphous powder.

¹H-NMR (200 MHz, CD₃OD) δ : 8.98 and 8.96 (d each, $J=2.0$ Hz, total 1H), 7.46 and 7.36 (d each, $J=2.0$ Hz, total 1H), 5.10–4.86 (m, 2H), 4.53 and 4.52 (d each, $J=9.0$ Hz, total 1H), 4.40 (m, 1H), 3.86 (m, 1H), 3.80–3.40 (m, 10H), 3.18 (dd, $J=14.4, 7.8$ Hz, 1H), 2.30–1.80 (m, 4H), 1.24 and 1.18 (d each, $J=6.4$ Hz, total 3H). $[\alpha]_D^{25} -69.7^\circ$ (c 0.51, H₂O). *Anal.* Calcd for C₂₁H₂₈N₆O₇S·1.7H₂O: C, 46.78; H, 5.87; N, 15.59; S, 5.95. Found: C, 46.78; H, 5.85; N, 15.59; S, 6.12.

General Procedure for the Synthesis of the C-Terminus Modified TRH Mimetics 54–59

Isopropyl [(4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolinate (**54**)

To an ice cooled solution of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**15**) (0.528 g, 3.64 mmol) and HOSu (0.419 g, 3.64 mmol) in DMF (10.0 mL), DCC (0.751 g, 3.64 mmol) was added and the mixture was stirred for 1 h. After the ice bath was removed, the reaction mixture was stirred continuously for 4 h, isopropyl [3-(thiazol-4-yl)-L-alanyl]-L-prolinate dihydrochloride (**50**) (1.04 g, 3.64 mmol) and triethylamine (2.03 mL, 14.6 mmol) were added and the mixture was stirred continuously for 16 h. The precipitate was filtrated off and the filtrate was concentrated under reduced pressure. The residue was purified by gel filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: H₂O/MeOH) and by silica gel column chromatography (eluent: CHCl₃/MeOH). The purified fractions were collected and lyophilized to afford the title compound **54** (0.640 g, 42%) as a white amorphous powder.

¹H-NMR (200 MHz, CD₃OD) δ : 8.99 and 8.95 (d each, $J=2.0$ Hz, total 1H), 7.44 and 7.36 (d each, $J=2.0$ Hz, total 1H), 5.00 (m, 3H), 4.40 (m, 1H), 4.34 (d, $J=8.6$ Hz, 1H), 3.93 (m, 1H), 3.66 (m, 1H), 2.30 (m, 1H), 2.00 (m, 3H), 1.28 (d, $J=6.2$ Hz, 6H), 1.20 (d, $J=6.6$ Hz, 3H). $[\alpha]_D^{25} -53.7^\circ$ (c 0.50, MeOH). *Anal.* Calcd for C₁₉H₂₆N₄O₆S·H₂O: C, 49.99; H, 6.18; N, 12.27; S, 7.02. Found: C, 50.01; H, 6.22; N, 12.41; S, 6.96.

In a similar manner, TRH mimetics **55–58** were prepared.

[(4*S*,5*S*)-5-Methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-proline (**55**)

The condensation of [3-(thiazol-4-yl)-L-alanyl]-L-proline dihydrochloride (**51**) (0.700 g, 1.65 mmol) and (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**15**) (0.239 g, 1.65 mmol) with HOSu (0.209 g, 1.82 mmol) and DCC (0.376 g, 1.82 mmol) in DMF (5.00 mL) yielded the title compound **55** (0.266 g, 41%) as a white amorphous powder.

¹H-NMR (300 MHz, CD₃OD) 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,

$J=8.4, 3.6\text{ Hz}$, 1H), 4.37 and 4.32 (d each, $J=8.7\text{ Hz}$, total 1H), 3.91 (m, 1H), 3.61 (m, 1H), 3.30 (m, 1H), 3.17 (dd, $J=14.7, 8.4\text{ Hz}$, 1H), 2.25 (m, 1H), 2.01 and 1.83 (m each, total 3H), 1.25 and 1.18 (d each, $J=6.9\text{ Hz}$, total 3H). $[\alpha]_{\text{D}}^{23} -52.0^\circ$ (c 1.0, H_2O). *Anal.* Calcd for $\text{C}_{16}\text{H}_{20}\text{N}_4\text{O}_6\text{S}\cdot 1.5\text{H}_2\text{O}$: 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.

tert-Butyl [(4*S*,5*S*)-5-Methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolyl-glycinate (**56**)

The condensation of [(4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-proline (**55**) (0.520 g, 1.31 mmol) and *tert*-butyl glycinate hydrochloride (**60**) (0.220 g, 1.31 mmol) with HOSu (0.150 g, 1.31 mmol), triethylamine (0.730 mL, 5.24 mmol) and DCC (0.300 g, 1.44 mmol) in DMF (50.0 mL) yielded the title compound **56** (0.530 g, 70%) as a white amorphous powder.

$^1\text{H-NMR}$ (300 MHz, CD_3OD) δ : 8.97 and 8.95 (d each, $J=2.1\text{ Hz}$, total 1H), 7.44 and 7.36 (d each, $J=2.1\text{ Hz}$, total 1H), 5.00 (t, $J=7.2\text{ Hz}$, 1H), 4.90 (m, 1H), 4.47 (dd, $J=8.4, 4.2\text{ Hz}$, 1H), 4.35 and 4.33 (d each, $J=8.7\text{ Hz}$, total 1H), 3.86 (m, 3H), 3.46 (m, 1H), 3.38 (dd, $J=14.7, 7.2\text{ Hz}$, 1H), 3.20 (dd, $J=14.7, 7.2\text{ Hz}$, 1H), 2.30–1.80 (m, 4H), 1.29 (s, 9H), 1.25 and 1.20 (d each, $J=6.6\text{ Hz}$, total 3H). $[\alpha]_{\text{D}}^{25} -66.2^\circ$ (c 1.0, MeOH). *Anal.* Calcd for $\text{C}_{22}\text{H}_{31}\text{N}_5\text{O}_7\text{S}\cdot 1.2\text{H}_2\text{O}$: C, 49.74; H, 6.34; N, 13.18; S, 6.04. Found: C, 49.68; H, 6.35; N, 13.33; S, 6.02.

Isopropyl [(4*S*,5*S*)-5-Methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolyl-glycinate (**57**)

The condensation of (4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carboxylic acid (**15**) (0.180 g, 1.24 mmol) and isopropyl [3-(4-thiazol)-L-alanyl]-L-prolyl-glycinate dihydrochloride (**52**) (0.590 g, 1.24 mmol) with HOSu (0.143 g, 1.24 mmol), triethylamine (0.690 mL, 4.96 mmol) and DCC (0.256 g, 1.24 mmol) in DMF (10.0 mL) yielded the title compound **57** (0.383 g, 57%) as a white amorphous powder.

$^1\text{H-NMR}$ (200 MHz, CD_3OD) δ : 8.97 and 8.96 (d each, $J=2.0\text{ Hz}$, total 1H), 7.45 and 7.36 (d each, $J=2.0\text{ Hz}$, total 1H), 4.99 (m, 2H), 4.59 (s, 2H), 4.47 (dd, $J=8.0, 4.0\text{ Hz}$, 1H), 4.33 (d, $J=8.4\text{ Hz}$, 1H), 3.93 (s, 2H), 3.85 (m, 1H), 3.35–3.25 (m, 2H), 3.20 (m, 1H), 2.30–1.80 (m, 4H), 1.24 (d, $J=6.4\text{ Hz}$, 6H), 1.25 and 1.21 (d each, $J=6.2\text{ Hz}$, total 3H). $[\alpha]_{\text{D}}^{23} -64.2^\circ$ (c 0.50, MeOH). *Anal.* Calcd for $\text{C}_{21}\text{H}_{29}\text{N}_5\text{O}_7\text{S}\cdot 1.1\text{H}_2\text{O}$: C, 48.94; H, 6.10; N, 13.59; S, 6.22. Found: C, 48.93; H, 6.00; N, 13.67; S, 6.03.

Benzyl [(4*S*,5*S*)-5-Methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolyl-glycinate (**58**)

The condensation of (4*S*,5*S*)-(5-methyl-2-oxooxazolidine-4-carboxylic acid (**15**) (0.210 g, 1.45 mmol) and benzyl [3-(4-thiazol)-L-alanyl]-L-prolyl-glycinate dihydrochloride (**53**) (0.710 g, 1.45 mmol) with HOSu (0.167 g, 1.45 mmol), triethylamine (0.810 mL, 5.81 mmol) and DCC (0.314 g, 1.52 mmol) in DMF (10.0 mL) yielded the title compound **58** (0.580 g, 74%) as a white amorphous powder.

$^1\text{H-NMR}$ (300 MHz, CD_3OD) δ : 8.97 and 8.95 (d each, $J=2.1\text{ Hz}$, total 1H), 7.42 and 7.36 (d each, $J=2.1\text{ Hz}$, total 1H), 7.35 (m, 5H), 5.18 (s, 2H), 4.95 (m, 2H), 4.47 (dd, $J=8.7, 4.2\text{ Hz}$, 1H), 4.33 (d, $J=8.7\text{ Hz}$, 1H), 4.07 (d, $J=17.7\text{ Hz}$, 1H), 3.99 (d, $J=17.7\text{ Hz}$, 1H), 3.80 (m, 1H), 3.60 (dd, $J=14.1, 6.9\text{ Hz}$, 1H), 3.35 (m, 1H), 3.22 (m, 1H), 2.20–1.90 (m, 4H), 1.25 and 1.21 (d each, $J=6.6\text{ Hz}$, total 3H). $[\alpha]_{\text{D}}^{23} -61.8^\circ$ (c 0.51, MeOH). *Anal.* Calcd for $\text{C}_{25}\text{H}_{29}\text{N}_5\text{O}_7\text{S}\cdot 0.3\text{H}_2\text{O}$: C, 54.69; H, 5.43; N, 12.76; S, 5.84. Found: C, 54.77; H, 5.57; N, 12.77;

S, 5.82.

[(4*S*,5*S*)-5-Methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolyl-glycine (**59**) To a solution of benzyl [(4*S*,5*S*)-5-methyl-2-oxooxazolidine-4-carbonyl]-[3-(thiazol-4-yl)-L-alanyl]-L-prolyl-glycinate (**58**) (0.500 g, 0.920 mmol) in THF (20.0 mL)- H_2O (20.0 mL), lithium hydroxide hydrate (0.193 g, 4.56 mmol) was added and the mixture was stirred for 1 h at room temperature. 1 M aqueous hydrochloric acid solution (4.56 mL, 4.56 mmol) was added to the mixture and concentrated under reduced pressure. To the residue, ethyl acetate (50.0 mL) and water (20.0 mL) were added and separated. The aqueous layer was washed with ethyl acetate (50.0 mL $\times 2$). The aqueous layer was concentrated *in vacuo*. The residue was purified by gel filtration chromatography (MCI GEL CHP-20P, 200 mL, eluent: $\text{H}_2\text{O}/\text{MeOH}$). The purified fractions were collected and lyophilized to afford the title compound **59** (0.218 g, 52%) as a white amorphous powder.

$^1\text{H-NMR}$ (200 MHz, CD_3OD) δ : 8.99 and 8.97 (d each, $J=2.0\text{ Hz}$, total 1H), 7.44 and 7.36 (d each, $J=2.0\text{ Hz}$, total 1H), 4.95 (m, 2H), 4.47 (t, $J=5.4\text{ Hz}$, 1H), 4.34 (d, $J=8.8\text{ Hz}$, 1H), 3.87 (d, $J=17.2\text{ Hz}$, 1H), 3.67 (d, $J=17.2\text{ Hz}$, 1H), 3.80–3.20 (m, 4H), 2.20–1.80 (m, 4H), 1.25 and 1.21 (d each, $J=6.2\text{ Hz}$, total 3H). $[\alpha]_{\text{D}}^{22.5} -69.2^\circ$ (c 0.51, H_2O). *Anal.* Calcd for $\text{C}_{18}\text{H}_{23}\text{N}_5\text{O}_7\text{S}\cdot 3.3\text{H}_2\text{O}$: C, 42.15; H, 5.82; N, 13.65; S, 6.25. Found: C, 41.88; H, 5.55; N, 13.76; S, 6.04.

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 (body weight 27–32 g) were purchased from SLC Japan Inc. (Shizuoka, Japan) 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. 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, Tokyo, Japan) before and after oral administration of TRH, **1** and TRH mimetics up to 7 h after a dose of 50, 5.0 and $10\text{ }\mu\text{mol/kg/mL}$, respectively. The antagonistic effect of the test drugs on reserpine-induced hypothermia was evaluated based on the area under the temperature–time curve after dosing ($\text{AUC}_{0-7\text{h}}$). All the mice used in the experiments were killed immediately after the last measurement.

TRH Receptor Binding Male Sprague-Dawley (SD) rats (body weight 300–400 g) 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 $40000\times g$ 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 [^3H]-[3-Me-His 2]-TRH (Daiichi Chemical, specific activity 82.5 Ci/mmol) and crude membrane prepa-

ration (approx. 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 with Whatman GF/C filter, and the filter was washed 3 times with the buffer, and then retained radioactively was counted with a liquid scintillation counter. The non-specific binding was measured in the presence of 10 μ M TRH (BACHEM, Switzerland). The saturation and inhibition binding studies were conducted with 0.125–8 nM and 2 nM of [3 H]-(3-Me-His 2)-TRH, respectively. The K_i values were calculated with the following formula: $K_i = IC_{50}/1 + (\text{ligand}/K_d)$.

PK Study in Rats Male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) at the age of 7 weeks. After quarantine for 1 week in the Animal Care Laboratory (Shionogi & Co., Ltd.), the rats (body weight 302–364 g) 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. For oral administration, 2 μ mol portions of TRH mimetics mixture as cassette dosing⁵⁶⁾ ($n=2$) were accurately weighed and suspended in the DMSO/0.5% aqueous 400 cP methyl cellulose solution = 1/4 v/v to obtain the dosing solution with the target concentration and dose of 2 μ mol/5 mL/kg. For iv administration, 1 μ mol portions of TRH mimetics mixture as cassette dosing were accurately weighed and dissolved in the DMSO/propylene glycol = 1/1 v/v to obtain the dosing solution with the target concentration and dose of 1 μ mol/1 mL/kg. Blood sampling times were 30, 60, 120, 240, 480, 1440 min after oral administration and 3, 10, 30, 60, 120, 240, 360 min after iv administration, respectively. Blood (approximately 0.3 mL) was collected from the jugular vein directly using a 20% v/v heparinized syringe containing 0.89 M ethylenediaminetetraacetic acid at the scheduled time after administration. The samples were immediately centrifuged by H-80R (Kokusan Co., Ltd., Saitama, Japan) in approximately 3000 rpm for 10 min at 4 °C 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 mimetics. Acetonitrile was added to the plasma with mixing in a tube with a mixer and centrifugation by Model 6000 (Kubota Co., Ltd., Osaka, Japan) in approximately 4500 rpm for 5 min at 8 °C to obtain the supernatant. The supernatant was injected into LC-MS/MS systems (electrospray ionization (ESI) positive mode), which was Triple Quad 5500 system (AB SCIEX Pte. Ltd., Tokyo, Japan) to determine the plasma concentration of TRH mimetics by non-std method.

In the brain distribution study, blood samples were collected 30 min after iv dosing through the abdominal aorta with the rat under isoflurane anesthesia and centrifuged for 15 min at 3000 rpm at 4 °C to obtain plasma. The plasma samples were transferred to separate tubes. The brain was removed and homogenized at a 3 ratio of tissue weight to mL of distilled water. Next, 200 μ L of plasma sample and 200 μ L of brain homogenate sample were transferred into separate tubes, and 200 μ L of blank brain homogenate was added to the plasma sample while 200 μ L of blank plasma was added to the

brain homogenate sample. The samples were mixed well and stored in a freezer until analysis. The samples were analyzed by LC-MS/MS. The B/P ratios (bKp) were determined from the peak area of the plasma sample (A_{plasma}) and the brain homogenate sample (A_{brain}):

$$\text{bKp} = (A_{\text{brain}} \times 4) / A_{\text{plasma}}$$

Protein Binding Assay Equilibrium dialysis membrane was soaked in purified water and phosphate buffered saline (PBS). This membrane was placed into a dialysis cell. TRH mimetics were accurately weighed and suspended in DMSO to prepare 1 μ mol/L solutions. 1.0 μ L of the solutions were respectively added to 249 μ L of rat sera to obtain the serum sample at 4.0 μ M. Next, 120 μ L of serum sample was placed into one side of the cell, and 120 μ L of PBS was placed into the other side of the cell ($n=2$). The cells were incubated by 100 rpm (CO $_2$ orbital shaker, As One corp.) at 37 °C for 22 h to 24 h in 5% CO $_2$ condition. After the incubation, 10 μ L of serum sample and 40 μ L of PBS sample were transferred into each tube. Next, 40 μ L of blank PBS was added to the serum sample, and 10 μ L of serum blank was added to PBS sample. The samples were mixed well and stored in a freezer until analysis. Acetonitrile/methanol = 1/1 v/v was added to samples with mixing in a tube with a mixer and centrifugation by Model 6000 (Kubota Co., Ltd.) in approximately 4500 rpm for 5 min at 8 °C to obtain the supernatants. The supernatants obtained by protein precipitation of samples were analyzed by LC-MS/MS. The analytical method was calibrated using a non-standard peak area. The unbound fraction in serum (f_u) was determined with peak area of serum sample (A_{serum}) and PBS sample (A_{PBS}) as follows:

$$f_u = A_{\text{PBS}} / (A_{\text{serum}} \times 4)$$

Serum Stability Assay The formulation for plasma protein binding were diluted with DMSO to prepare 20 μ mol/L solutions, and 10 μ L of the solutions were respectively added to 40 μ L of serum and mixed to obtain the serum sample at 4 μ mol/L. The serum samples were incubated for 4 and 24 h at 37 °C in 5% CO $_2$ condition. Acetonitrile/methanol = 1/1 v/v was added to samples with mixing in a tube with a mixer and centrifugation by Model 6000 (Kubota Co., Ltd.) in approximately 4500 rpm for 5 min at 8 °C to obtain the supernatants. The supernatants obtained by protein precipitation of serum samples were analyzed by LC-MS/MS. The analytical method was calibrated using a non-standard peak area. The remaining % at 24 h was calculated by comparing the peak area at 0 and 24 h. The half-life time ($t_{1/2}$) in serum was calculated assuming of first order degradation.

$$t_{1/2} = 0.693 \times \text{reaction time} / \ln(\text{remaining \%})$$

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

The synthetic procedure and analytical data for intermediates **16–26**, **34–43** and **45–53**.

Rectal temperature–time graphs of TRH mimetics **1**, **10**, **11**, **13**, **28–32**, **54–57**, **59** and detail calculation method of antihypothermic effects.

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