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Synthesis and Biology of New Thyrotropin-Releasing Hormone (TRH) Analogues†

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Abstract—We report synthesis and biological activities of several thyrotropin-releasing hormone (TRH) analogues in which the N-terminal pyroglutamic acid residue has been replaced with various carboxylic acids and the central histidine is modified with substituted-imidazole derivatives.

Introduction

Thyrotropin-releasing hormone (TRH, L-pGlu-L-His-L-Pro-NH_2), a tripeptide synthesized in the hypothalamus,[1] operates in the anterior pituitary to control levels of TSH (thyroid-stimulating hormone) and prolactin.[2] This peptide was the first hypothalamic releasing factor characterized, establishing the fundamental proof for the existence of a neuroendocrine regulation of pituitary function by hypothalamic neuronal structures.[1,3] The same peptide is found in many other tissues and appears to be involved in a wide variety of physiological activities.[4] Elucidation of its mechanism of action, identification of critical features of the molecule, separation of its multiple activities through design of selective analogues and affinity labels, and stabilization to enzymatic degradation have been elusive goals for 30 years.

Results and Discussion

Isolation and cloning of the pituitary receptor, as well as replacement by point mutation of predicted key amino acids, have permitted Perlman et al.[5] to formulate the first model of the TRH peptide–receptor complex. Since the first residue of TRH, pyroglutamic acid, is known to be responsible for at least half of the peptide's binding energy, its role was first investigated by Perlman et al.[5] Removal of the carbonyl group of the five-membered ring (replacement with proline) results in loss of 100,000-fold in binding affinity (based on loss of activity). An equivalent loss in affinity is observed on replacement of Tyr-106 by Phe in helix 3 of the receptor. It is possible, therefore, that a strong H-bond exists between the hydroxyl of Tyr-106 and the ring carbonyl group. Parallel manipulations show that the ring nitrogen of pyroglutamic acid forms a critical H-bond with Asparagine-110 of helix 3.[6] However, Tashjian et al.[7] have concluded by single residue mutations at the other amino acids that the role of Tyr106 in TRH binding is indirect, and proposed a hydrogen bonding interaction of Asn-289 in the third extracellular loop (EL3) with lactam moiety of the pyroglutamyl ring of TRH.

In a paper published recently,[8] we find that retention of the carbonyl, but replacement of the ring N–H group of pGlu by a methylene group, provides an analogue (RJ-601) whose binding affinity and signal-transducing potency are approximately 100-fold less than those of TRH itself. Hence, we concluded that the H-bond interaction to Tyr-106 is considerably more important than that to Asn-110, and the loss of NH binding is partially compensated by the strong H-bonding ability of the ketonic carbonyl group. To further validate this hypothesis, and in continuation of our earlier work, this paper describes synthesis and biological activities of several TRH analogues, where pGlu has been replaced with carboxylic acids (**2**–**5**) of varying hydrogen-bond donating abilities (Fig. 1).

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Development of synthetic methodologies for the ring-substituted histidines enabled Cohen et al. to prepare first highly selective cardiovascular analogue which was nearly devoid of endocrine effects.[9] Furthermore, 1-CH_3-Im-TRH is found to bind 7–10 times as tightly as TRH to its receptor.[10] This encouraged us to synthesize substituted-imidazole TRH derivatives (RJ-640 and RJ-641) where central histidine residue is replaced with its 1-methyl counterpart,[11] and pGlu is replaced with its (1*S*)- and (1*R*)-desaza counterpart. On the other hand, easy availability to previously inaccessible 2-iodo-L-histidine[12] enabled us to synthesize TRH analogue RJ-504.

Synthesis

Cyclopentanecarboxylic acid (**2**) was purchased from Aldrich Chemicals Ltd (Milwaukee, WI, USA). 3-Oxocyclopentane-1-carboxylic acid (**3**) was prepared in four step from 4-vinylcyclohexene following previously published procedure.[13] Resolution of racemic carboxylic acid (**3**) with brucine hydrate readily afforded (1*R*)- and (1*S*)-3-oxocyclopentane-1-carboxylic acids (**3**) as described earlier (Fig. 1).[14] *O*-Benzyl protected derivatives of oxime (**4**) were synthesized by the treatment of (1*R*)- and (1*S*)-3-oxocylopentane-1-carboxylic acids (**3**) with *O*-benzylhydroxylamine and pyridine in ethanol by method previously described.[15] Both enantiomers of 3-oxocyclopentane-1-carboxylic acid (**3**) and its oxime derivative (**4**) were incorporated into the peptide. Whereas, *N*-hydroxy-L-proline (**5**) was synthesized from L-proline in three steps according to published procedure.[16] Esterification of 1-methyl-L-histidine[11] and 2-iodo-L-histidine[12] using anhydrous methanolic hydrogen chloride gave methyl ester derivatives. The latter compounds on treatment with 2*M* solutions of ammonia in chloroform afforded 1-methyl-L-histidine methyl ester (**6**) and 2-iodo-L-histidine methyl ester (**7**). Carboxylic acids (**1**–**4**) on reaction with 2,4,5-trichlorophenol in the presence of DCC in DMF at −10 °C gave 2,4,5-trichlorophenyl active esters (**8**–**11**) in excellent yield (Schemes 1 and 2).

All tripeptides were assembled using the solution phase peptide chemistry methodologies. Thus, 2,4,5-trichlorophenyl esters (**8**–**11**) on reaction with histidine methyl ester derivatives in anhydrous ethyl acetate for 24 h at ambient temperature afforded crude protected dipeptides, which were purified by column chromatography on silica gel using EtOAc/CH_3OH (94:6). Hydrolysis of the methyl ester group in dipeptides with 0.5 N NaOH in methanol (40 min at room temperature) gave crude dipeptides (**12**–**20**). The solution of latter compounds on pH adjustment to 6.0 with Dowex ion exchange resin (50×2-200, H^+ form) provided free dipeptides (**12**–**20**). Finally, TRH analogues were conveniently prepared by the treatment of L-ProNH_2 with the dipeptides (**12**–**20**) in the presence of DCC and HOBt in DMF. All tripeptides were purified by flash column chromatography on silica gel with $CHCl_3$/CH_3OH (1:9) as eluant (Scheme 1). *O*-Benzyl protection of the 3-oxoiminocyclopentyl ring in tripeptides was removed by catalytic hydrogenation with 10% Pd on carbon and H_2 in ethanol for 2 h at room temperature and pressure to produce TRH analogues (RJ-644 and RJ-645). However, an alternative synthetic route was followed for the synthesis of RJ-643. Thus, L-His-L-Pro-NH_2 (**21**)[17] on reaction with *N*-hydroxy-L-proline (**5**) in the presence of DCC and HOBt in DMF afforded RJ-643, which was purified by flash column chromatography on silica gel with $CHCl_3$/CH_3OH/NH_4OH (5:4:1) as eluant.

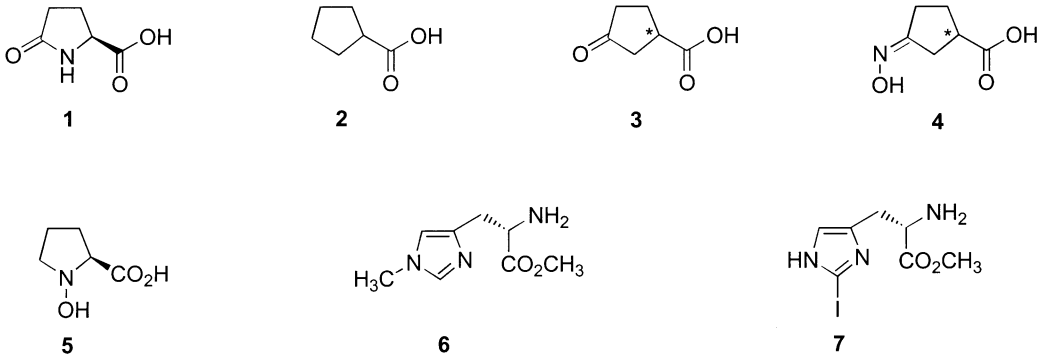
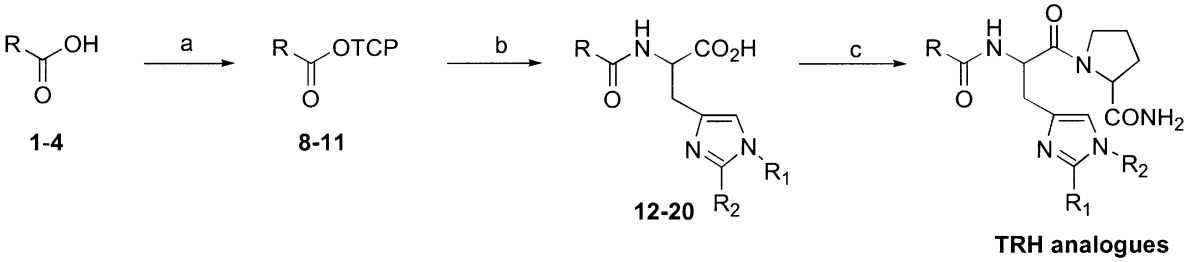


Figure 1.

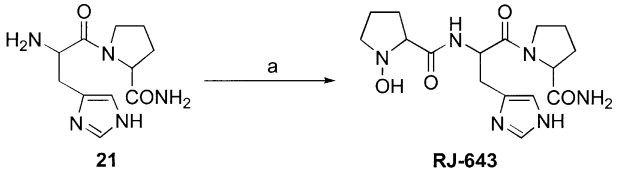


Scheme 1. (a) 2,4,5-trichlorophenol, DCC, DMF; (b) i. H-His(R_1,R_2)-OMe, EtOAc; ii. 0.5N NaOH, MeOH, ion-exchange (H^+); (c) Pro-NH_2, DCC, HOBt, DMF.

Biological activity

All TRH analogues were tested for their ability to activate thyrotropin-releasing hormone receptor (TRH-R subtype 1) and were compared to TRH (Table 1). The EC_{50}s (activation potencies) for the second messenger inositol phosphate formation, and the IC_{50}s (binding affinities) were obtained according to previously reported procedures.[5,18] The data revealed that the affinities and potencies of the analogues RJ-644 and RJ-645 [replacement of pGlu with (1*S*)- and (1*R*)-(3-oximinocyclopentyl)] were markedly lower than that of the TRH. Similarly, peptide analogue RJ-643, synthesized to check the hydrogen bonding interaction capabilities within the receptor, where pGlu has been replaced with *N*-hydroxy-L-proline exhibited markedly lowered affinity and potency (Table 1). Furthermore, the cyclopentyl derivative (RJ-592), where lactam group of pGlu has been replaced with methylene groups to further confirm the importance of the lactam group in binding to the TRH receptor, exhibited an even greater loss in affinity and potency (Table 1).

As reported earlier,[8] tripeptide RJ-601 exhibited binding affinity and signal-transducing potency, which are approximately 100-fold less than those of TRH itself. The incorporation of methyl group at the N-1 position of imidazole ring (RJ-641, RJ-640, $R_1 = CH_3$) caused a 10-fold increase in binding affinity and potency that was independent of the changes at the pGlu residue. The data clearly indicated that the maximal extent of stimulation of inositol phosphate formation and binding affinities for peptides RJ-641 and RJ-640 were approximately 10-fold higher than those of RJ-600 and RJ-601. These observations are consistent with earlier results that the affinity and potency of 1-CH_3-Im-TRH are approximately 10-fold higher than those of TRH.[10] Lastly; the analogue with iodine at the R_2-position of the imidazole ring (RJ-504) exhibited a markedly lowered affinity and potency. These findings are consistent with previous observations that radioiodinated TRH was a poor ligand for the receptor.



Scheme 2. (a) *N*-hydroxy-L-Pro-OH, DCC, HOBt, DMF.

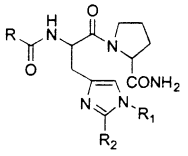
Conclusions

In conclusion, we have demonstrated that substitution of the pGlu with other hydrogen-bond donating moieties in the peptide does not stop the peptides from binding and activating TRH-R. We believe that our continuing efforts to accumulate data on the size, geometry and flexibility of the pyroglutamate-binding pocket will permit us to design irreversible affinity labels to form covalent bonds to Tyr-106 and Asn-110. At the same time, we are trying to rationalize the requirements for the H-bonding to histidine and its analogues. Ultimately, we hope to settle the controversy between the divergent formulations of TRH-receptor binding, as well as to design selective analogues, which may be more useful clinically than TRH itself.

Experimental

Melting points were determined with a capillary apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Bruker Avance DPX (300 MHz) spectrometer using TMS as internal reference. Mass spectra were recorded on Shimadsu GCMS-QP 5000 spectro-

Table 1. Binding affinities IC_{50} (nM) and activation potencies EC_{50} (nM) of TRH-R receptors by TRH analogues



Entry	*R*	R_1	R_2	IC_{50}[a]	EC_{50}[b]
RJ-504	(2*S*)-PyroGlu	H	I	14,000	950
RJ-592	Cyclopentyl	H	H	$>10^6$	$>10^6$
RJ-600	(1*S*)-(3-Oxocyclopentyl)	H	H	13,500±5000	980±440
RJ-601	(1*R*)-(3-Oxocyclopentyl)	H	H	1950±300	100±40
RJ-640	(1*S*)-(3-Oxocyclopentyl)	CH_3	H	3100±460	250±300
RJ-641	(1*R*)-(3-Oxocyclopentyl)	CH_3	H	170±30	16±1
RJ-643	(2*S*)-1-Hydroxyproline	H	H	>300,000	70,000±35,000
RJ-644	(1*S*)-(3-Oximinocyclopentyl)	H	H	160,000	50,000
RJ-645	(1*R*)-(3-Oximinocyclopentyl)	H	H	46,000±9500	6300±4400
TRH	(2*S*)-PyroGlu	H	H	16±3	0.50±0.10

All data are means ±SD of duplicate determinations in two or three experiments.[a]For binding, cells were incubated with 1 nM [^{3}H]1-CH_3-Im-TRH in the absence or presence of various doses of unlabeled TRH analogues for 1 h at 37 °C.
[b]For activation, cells prelabeled with *myo*-[^{3}H]inositol were incubated with various doses of TRH analogues for 1 h at 37 °C, and inositol phosphate formation was measured. Maximal extents of stimulation were similar for all analogues. Experiments were performed with intact AtT-20 mouse pituitary tumor cells stably expressing TRH receptors.

meter, whereas ESI (Electron Spray Ionization) spectra were recorded on a LCMS Finnigan Mat LCQ spectrometer. Optical rotations were recorded on a Perkin–Elmer 241 MC Polarimeter. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA, USA. All chromatographic purification was performed with silica gel 60 (230–400 mesh), whereas all TLC (silica gel) development was performed on silica gel coated (Merck Kiesel 60 F254, 0.2 mm thickness) sheets. Following abbreviations are used: pGlu, pyroglutamic acid; DCC, 1,3-dicyclohexylcarbodiimide; DCU, 1,3-dicyclohexylurea; DMF, *N*,*N*-dimethylformamide; Boc, *tert*-butyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; EtOAc, ethyl acetate; DIEA, *N*,*N*-diisopropylethylamine; Im, imidazole; MeOH, methanol; OTCP, 2,4,5-trichlorophenyl ester.

General procedure for the synthesis of substituted L-histidine methyl esters (6–7)

To a solution of ring-substituted histidine derivative (100 mmol) in methanol (100 mL) cooled to 0 °C was bubbled HCl gas for 45 min. The solution was allowed to stand overnight at room temperature. The solvent was evaporated under reduced pressure to afford the salts of the substituted L-histidine methyl ester as semi-solid. A 2 M solution of ammonia in $CHCl_3$ (10 mL) was then added to the dihydrochloride salt, and the reaction mixture was allowed to stand for 30 min at room temperature. Separated solid filtered and complete evaporation of the filtrate under reduced pressure gave the substituted L-histidine methyl esters.

1-Methyl-L-histidine methyl ester (6). Yield: 95%; oil; ^{1}H NMR ($CDCl_3$) δ 2.60–2.81 (m, 2H, CH_2), 3.53 (s, 3H, N–CH_3), 3.66 (s, 3H, OCH_3), 4.10 (m, 1H, CH), 7.16 (s, 1H, 5-H), 7.37 (s, 1H, 2-H); CIMS (NH_3) *m*/*z* 184 (M + 1); analysis for $C_8H_{13}N_3O_2$ (183.2), calcd C, 52.45; H, 7.15; N, 22.94; found C, 52.44; H, 7.09; N, 23.07.

2-Iodo-L-histidine methyl ester (7). Yield 90%; oil; ^{1}H NMR ($CDCl_3$) δ 2.74–2.90 (m, 2H, CH_2), 3.66 (s, 3H, OCH_3), 4.13 (m, 1H, CH), 7.55 (s, 1H, 5-H); CIMS (NH_3) *m*/*z* 296 (M + 1); analysis for $C_7H_{10}IN_3O_2$ (295.1), calcd C, 28.49; H, 3.42; N, 14.24; found C, 28.67; H, 3.11; N, 14.33.

General procedure for the synthesis of 2,4,5-trichlorophenyl esters (8–11)

A mixture of DCC (1 mmol) and 2,4,5-trichlorophenol (1 mmol) in DMF (50 mL) was cooled to −10 °C in an ice-salt bath and treated dropwise with a solution of carboxylic acid (**1**–**4**, 1 mmol) in DMF (10 mL). The reaction mixture was allowed to warm to room temperature and was stirred overnight. Removal of the solvent under reduced pressure afforded a white solid. The solid was triturated with ethyl acetate (50 mL), and the dicyclohexylurea was removed by filtration. Filtrate was evaporated under reduced pressure to afford oil. Flash chromatography on silica gel using EtOAc/hexanes (1:9) gave active esters (**8**–**11**) in excellent yield.

8 [R = (2*S*)-pyroGlu]. Yield: 90%; mp: 71–72 °C; ^{1}H NMR ($CDCl_3$) δ 2.5 (m, 4H, 2×CH_2), 4.55 (m, 1H, CH), 7.30 (s, 1H, Ar–H), 7.56 (s, 1H, Ar–H); CIMS (NH_3) *m*/*z* 310 (M + 1); analysis for $C_{11}H_8Cl_3NO_3$ (308.5); calcd C, 42.82; H, 2.61; N, 4.54; found C, 42.88; H, 2.50; N, 4.33.

9 [R = cyclopentyl]. Yield: 88%; mp: 66 °C; ^{1}H NMR ($CDCl_3$) 1.94 (m, 4H, 2×CH_2), 2.10 (m, 4H, 2×CH_2), 2.51 (m, 1H, CH), 7.24 (s, 1H, Ar–H), 7.27 (s, 1H, Ar–H), CIMS (NH_3) *m*/*z* 295 (M + 1); analysis for $C_{12}H_{11}Cl_3O_2$ (293.6), calcd C, 49.09; H, 3.78; found C, 48.90; H, 4.00.

10a [R = (1*S*)-(3-oxocyclopentyl)]. Yield: 81%; mp 55–56 °C; ^{1}H NMR ($CDCl_3$) δ 2.3–2.73 (m, 6H, 3×CH_2), 4.44 (m, 1H, CH), 7.29 (s, 1H, Ar–H), 7.51 (s, 1H, Ar–H); CIMS (NH_3) *m*/*z* 309 (M + 1); analysis for $C_{12}H_9Cl_3O_3$ (307.6), calcd C, 46.86; H, 2.95; found C, 46.99; H, 3.23; $[\alpha]_D^{25}$ −19.0° (*c* 1.2, $CHCl_3$).

10b [R = (1*R*)-(3-oxocyclopentyl)]. Yield: 63%; mp 56–57 °C; analysis for $C_{12}H_9Cl_3O_3$ (307.56), calcd C, 46.86; H, 2.95; found C, 47.13; H, 2.88; $[\alpha]_D^{25}$ +18.8° (*c* 1.95, $CHCl_3$).

11a [R = *O*-benzyl oxime of (1*S*)-3-oxocyclopentyl]. Yield: 73%; oil; ^{1}H NMR ($CDCl_3$) δ 2.12 (m, 2H, CH_2), 2.40 (m, 4H, 2×CH_2), 3.99 (m, 1H, CH), 5.20 (s, 1H, CH_2), 7.30 (m, 3H, Ar–H), 7.50 (m, 2H, Ar–H); CIMS (NH_3) *m*/*z* 414 (M + 1); analysis for $C_{19}H_{16}Cl_3NO_3$ (412.7), calcd C, 55.30; H, 3.91; N, 3.39; found C, 55.63; H, 3.77; N, 3.55.

11b [R = *O*-benzyl oxime of (1*R*)-3-oxocyclopentyl]. Yield: 37%; oil; analysis for $C_{19}H_{16}Cl_3NO_3$ (412.7), calcd C, 55.30; H, 3.91; N, 3.39; found C, 55.29; H, 3.98; N, 3.44.

General method for the synthesis of dipeptides (12–20)

A solution of 2,4,5-trichlorophenyl ester (**8**–**11**, 1 mmol) in EtOAc (10 mL) was added drop wise to substituted L-histidine methyl ester (**6**–**7**) or L-histidine methyl ester (1 mmol) in EtOAc (50 mL) during 15 min, and reaction mixture was stirred at ambient temperature for 24 h. Complete removal of the solvent in vacuo gave crude product. Flash column chromatography on silica gel using a solvent system of EtOAc/CH_3OH (94:6) provided dipeptide methyl ester. Pure dipeptide methyl ester (1 mmol) was then dissolved in a mixture of MeOH (50 mL) and 0.5 N NaOH (40 mL). The solution was stirred at ambient temperature for 30 min, and water (35 mL) was added to the solution. The pH of the solution was then adjusted to 6.0 with Dowex (50×2–200, H^+ form) ion exchanger. The resin was removed by filtration, and the filtrate was evaporated under vacuum to afford crude product. Crystallized from methanol/ethanol/ether (1:1:1).

12 [R = (2*S*)-pyroGlu, $R_1 = R_2 = H$]. Yield: 72%; mp 218 °C (dec); ^{1}H NMR (D_2O) δ 1.83 (m, 2H, CH_2), 2.31 (m, 2H, CH_2), 3.1 (m, 2H, CH_2), 4.18 (m, 1H, CH), 4.41

(m, 1H, CH), 7.08 (s, 1H, 5-H), 8.30 (s, 1H, 2-H); ESIMS m/z 267 (M + 1); analysis for $C_{11}H_{14}N_4O_4$ (266.3), calcd C, 42.62; H, 5.30; N, 21.04; found C, 42.44; H, 5.72; N, 20.88; $[\alpha]_D^{25}$ +4.2° (*c* 1, H_2O).

13 [R = cyclopentyl, $R_1 = R_2 = H$]. Yield: 80%; mp 176–178 °C (dec); ^1H NMR (D_2O) δ 1.76 (m, 4H, 2×CH_2), 2.31 (m, 4H, 2×CH_2), 3.01 (m, 2H, CH_2), 4.10 (m, 2H, 2×CH), 7.03 (s, 1H, 5-H), 8.26 (s, 1H, 2-H); ESIMS m/z 252 (M + 1); analysis for $C_{12}H_{17}N_3O_3$ (251.3); calcd C, 57.36; H, 6.82; N, 16.72; found C, 57.49; H, 6.84; N, 16.98.

14 [R = (1*S*)-(3-oxocyclopentyl), $R_1 = R_2 = H$]. Yield: 77%; mp: 185–186 °C (dec); ^1H NMR (D_2O) δ 1.75–2.61 (m, 6H, 3×CH_2), 3.11 (m, 2H, CH_2), 4.01 (m, 2H, 2×CH), 7.13 (s, 1H, 5-H), 8.36 (s, 1H, 5-H); ESIMS m/z 266 (M + 1); analysis for $C_{12}H_{15}N_3O_4$ (265.3); calcd C, 54.33; H, 5.70; N, 15.84; found C, 54.21; H, 5.89; N, 15.88.

15 [R = (1*R*)-(3-oxocyclopentyl), $R_1 = R_2 = H$]. Yield: 65%; mp: 192–193 °C (dec); ^1H NMR (D_2O) δ 1.80–2.61 (m, 6H, 3×CH_2), 3.04 (m, 2H, CH_2), 4.13 (m, 2H, 2×CH), 7.03 (s, 1H, 5-H), 8.29 (s, 1H, 5-H); ESIMS m/z 266 (M + 1); analysis for $C_{12}H_{15}N_3O_4$ (265.3); calcd C, 54.33; H, 5.70; N, 15.84; found C, 54.49; H, 5.73; N, 15.79.

16 [R = (1*S*)-(3-oxocyclopentyl), $R_1 = CH_3$, $R_2 = H$]. Yield: 86%; mp: 201–202 °C (dec); ^1H NMR (D_2O) δ 1.70 (m, 2H, CH_2), 2.28 (m, 4H, 2×CH_2), 2.89 (m, 1H, CH), 3.09 (m, 1H, CH), 3.71 (s, 3H, N–CH_3), 4.40 (m, 1H, CH), 7.08 (s, 1H, 5-H), 8.27 (s, 1H, 2-H); ESIMS m/z 280 (M + 1); analysis for $C_{13}H_{17}N_3O_4$ (279.3), calcd C, 55.91; H, 6.14; N, 15.05; found C, 56.21; H, 6.03; N, 15.21.

17 [R = (1*R*)-(3-oxocyclopentyl), $R_1 = CH_3$, $R_2 = H$]. Yield: 79%; mp: 196–198 °C (dec); ^1H NMR (D_2O) δ 1.73 (m, 2H, CH_2), 2.33 (m, 4H, 2×CH_2), 2.92 (m, 1H, CH), 3.11 (m, 1H, CH), 3.76 (s, 3H, N–CH_3), 4.38 (m, 1H, CH), 7.03 (s, 1H, 5-H), 8.20 (s, 1H, 2-H); ESIMS m/z 280 (M + 1); analysis for $C_{13}H_{17}N_3O_4$ (279.3), calcd C, 55.91, H, 6.14, N, 15.05, found C, 56.07; H, 6.34; N, 15.09.

18 [R = *O*-benzyl oxime of (1*S*)-(3-oxocyclopentyl), $R_1 = R_2 = H$]. Yield: 88%; mp: 212–214 °C; ^1H NMR (D_2O) δ 1.69 (m, 2H, CH_2), 2.31 (m, 4H, 2×CH_2), 2.93 (m, 1H, CH), 3.08 (m, 1H, CH), 4.30 (m, 1H, CH), 5.17 (s, 2H, CH_2), 7.05 (s, 1H, 5-H), 7.27 (m, 3H, Ar–H), 7.59 (m, 2H, Ar–H), 8.30 (s, 1H, 2-H); ESIMS m/z 371 (M + 1); analysis for $C_{19}H_{22}N_4O_4$ (370.4); calcd C, 61.61; H, 5.99; N, 15.13; found C, 61.33; H, 5.76; N, 15.14.

19 [R = *O*-benzyl oxime of (1*R*)-(3-oxocyclopentyl), $R_1 = R_2 = H$]. Yield: 65%; mp 196–198 °C; ^1H NMR (D_2O) δ 1.72 (m, 2H, CH_2), 2.40 (m, 4H, 2×CH_2), 2.98 (m, 1H, CH), 3.12 (m, 1H, CH), 4.28 (m, 1H, CH), 5.20 (s, 2H, CH_2), 7.10 (s, 1H, 5-H), 7.30 (m, 3H, Ar–H), 7.60 (m, 2H, Ar–H), 8.33 (s, 1H, 2-H); ESIMS m/z 371 (M + 1); analysis for $C_{19}H_{22}N_4O_4$ (370.4); calcd C, 61.61; H, 5.99; N, 15.13; found C, 61.66; H, 6.22; N, 14.97.

20 [R = pyroGlu, $R_1 = H$, $R_2 = I$]. Yield: 99%; mp 225–227 °C; ^1H NMR (D_2O) δ 1.74 (m, 2H, CH_2), 2.29 (m, 4H, 2×CH_2), 2.82 (m, 1H, CH), 3.05 (m, 1H, CH), 4.13 (m, 1H, CH), 4.36 (m, 1H, CH), 6.82 (s, 1H, 5-H); ESIMS m/z 393 (M + 1); analysis for $C_{11}H_{13}IN_4O_4$ (392.2); calcd C, 33.69; H, 3.34; N, 14.29; found C, 33.77; H, 3.36; N, 14.34; $[\alpha]_D^{25}$ +7.8° (*c* 0.4, MeOH).

General procedure for the synthesis of TRH analogues

Dipeptide (**12**–**20**, 1 mmol) was dissolved in DMF (25 mL), and HOBt (1 mmol) was added to the solution followed by addition of DCC (1 mmol) under cooling at 0 °C. Stirring of the reaction mixture continued for another 30 min at 0 °C. L-Prolineamide (1.2 mmol) was then added to this solution in one portion. The reaction mixture was allowed to warm to room temperature and was stirred for 48 h. Separated DCU was removed by filtration, and filtrate evaporated in vacuo to afford crude product. Flash column chromatography on silica gel using $CHCl_3/CH_3OH$ (1:9) as the solvent system gave pure TRH analogue as white solid. The homogeneity of peptide analogues on TLC was assessed using $CHCl_3/CH_3OH/NH_4OH$ (4:5:1) as mobile phase.

Spectral data for RJ-504. Yield: 40%; ^1H NMR (CD_3OD) δ 1.75 (m, 4H, 2×CH_2), 2.50 (m, 6H, 3×CH_2), 2.78 (m, 2H, CH), 3.10 (m, 2H, CH), 4.01 (m, 1H, CH), 6.88 (s, 1H, 5-H), ESIMS m/z 489 (M + 1); analysis for $C_{16}H_{21}IN_6O_4$ (488.3); calcd C, 39.36; H, 4.33; N, 17.21; found C, 39.66; H, 4.47; N, 17.04; $[\alpha]_D^{25}$ −30.4° (*c* 1.2, MeOH); R_f 0.50 (one spot).

Spectra data for RJ-592. Yield: 44%; ^1H NMR (CD_3OD) δ 1.18 (m, 8H, 3×CH_2), 2.37 (m, 8H, 4×CH_2), 2.56 (m, 1H, CH), 2.80 (m, 1H, CH), 2.90 (m, 1H, CH), 3.50 (m, 1H, CH), 4.40 (m, 1H, CH), 6.76 (s, 1H, 5-H), 7.53 (s, 1H, 2-H); ESIMS m/z 348 (M + 1); analysis for $C_{17}H_{25}N_5O_3$ (347.4); calcd C, 58.77; H, 7.25; N, 20.16; found C, 58.59; H, 6.89; N, 20.43; $[\alpha]_D^{25}$ −25.4° (*c* 1.2, MeOH); R_f 0.63 (one spot).

Spectral data for RJ-600. Yield: 50%; ^1H NMR (CD_3OD) δ 1.86 (m, 6H, 3×CH_2), 2.17 (m, 6H, 3×CH_2), 2.94 (m, 2H, CH_2), 3.27 (m, 1H, CH), 3.67 (m, 1H, CH), 4.30 (m, 1H, CH), 6.86 (s, 1H, 5-H), 7.55 (s, 1H, 2-H); ESIMS m/z 362 (M + 1); analysis for $C_{17}H_{23}N_5O_4$ (361.4); calcd, C, 56.50; H, 6.41; N, 19.38; found, C, 56.34; H, 6.45; N, 19.65; $[\alpha]_D^{25}$ −46.1° (*c* 1.15, MeOH); R_f 0.55 (one spot).

Spectral data for RJ-601. Yield: 28%; ^1H NMR (CD_3OD) δ 1.85 (m, 6H, 3×CH_2), 2.15 (m, 6H, 3×CH_2), 2.94 (m, 2H, CH_2), 3.26 (m, 1H, CH), 3.65 (m, 1H, CH), 4.31 (m, 1H, CH), 6.85 (s, 1H, 5-H), 7.51 (s, 1H, 2-H); ESIMS m/z 362 (M + 1); analysis for $C_{17}H_{23}N_5O_4$ (361.4); calcd, C, 56.50; H, 6.41; N, 19.38; found, C, 56.76; H, 6.76; N, 19.34; $[\alpha]_D^{25}$ −26.5° (*c* 1.14, MeOH); R_f 0.55 (one spot).

Spectral data for RJ-640. Yield: 23%; ^{1}H NMR (CD_3OD) δ 1.88 (m, 6H, 3×CH_2), 2.17 (m, 6H, 3×CH_2), 2.91 (m, 2H, CH_2), 3.30 (m, 1H, CH), 3.63 (m, 1H, CH), 3.77 (s, 3H, N–CH_3), 4.28 (m, 1H, CH), 6.90 (s, 1H, 5-H), 7.59 (s, 1H, 2-H); ESIMS *m*/*z* 376 (M + 1); analysis for $C_{18}H_{25}N_5O_4$ (375.4); calcd C, 57.59; H, 6.71; N, 18.65; found C, 57.89; H, 6.93; N, 18.54; $[\alpha]_D^{25}$ −52.3° (*c* 1.2, MeOH); R_f 0.60 (one spot).

Spectral data for RJ-641. Yield: 36%; ^{1}H NMR (CD_3OD) δ 1.89 (m, 6H, 3×CH_2), 2.14 (m, 6H, 3×CH_2), 2.99 (m, 2H, CH_2), 3.36 (m, 1H, CH), 3.61 (m, 1H, CH), 3.80 (s, 3H, N–CH_3), 4.21 (m, 1H, CH), 6.91 (s, 1H, 5-H), 7.58 (s, 1H, 2-H); ESIMS *m*/*z* 376 (M + 1); analysis for $C_{18}H_{25}N_5O_4$ (375.4); calcd C, 57.59; H, 6.71; N, 18.65; found C, 57.47; H, 6.68; N, 18.58; $[\alpha]_D^{25}$ −33.5° (*c* 1.2, MeOH); R_f 0.60 (one spot).

Spectral data for RJ-644. Yield: 35%; ^{1}H NMR (CD_3OD) δ 1.87 (m, 6H, 3×CH_2), 2.11–2.98 (m, 8H, 4×CH_2), 3.38 (m, 1H, CH), 3.66 (m, 1H, CH), 4.33 (m, 1H, CH), 6.87 (s, 1H, 5-H), 7.56 (m, 1H, 2-H); ESIMS *m*/*z* 377 (M + 1); analysis for $C_{17}H_{24}N_6O_4$ (376.4); calcd, C, 54.24; H, 6.43; N, 22.33; found, C, 54.03; H, 6.12; N, 22.57; $[\alpha]_D^{25}$ −57.8° (*c* 1.2, MeOH); R_f 0.37 (one spot).

Spectral data for RJ-645. Yield: 43%; ^{1}H NMR (CD_3OD) δ 1.79 (m, 6H, 3×CH_2), 2.10–2.96 (m, 8H, 4×CH_2), 3.35 (m, 1H, CH), 3.69 (m, 1H, CH), 4.29 (m, 1H, CH), 6.86 (s, 1H, 5-H), 7.57 (m, 1H, 2-H); ESIMS *m*/*z* 377 (M + 1); analysis for $C_{17}H_{24}N_6O_4$ (376.4); calcd C, 54.24; H, 6.43; N, 22.33; found C, 53.97; H, 6.57; N, 22.64; $[\alpha]_D^{25}$ −50.3° (*c* 1.2, MeOH); R_f 0.55 (one spot).

Procedure for the synthesis of tripeptide RJ-643. *N*-Hydroxy-L-proline (1 mmol) was dissolved in DMF (20 mL), and HOBt (1.2 mmol) was added to the solution followed by addition of DCC (1.2 mmol) under cooling at 0 °C. The mixture was stirred at the same temperature for 6 h. A solution of the L-histidine-L-proline-NH_2[17] (1.2 mmol) in DMF (15 mL) was then added at once, and the reaction mixture was stirred at 5 °C overnight. After the resulting precipitate was filtered off, the filtrate was evaporated to dryness under reduced pressure. The residue was subjected to column chromatography with chloroform/methanol/ammonia (50:40:10) as eluant. The required fractions were pooled and concentrated under reduced pressure to afford RJ-643 as powder. Yield: 21%; ^{1}H NMR (CD_3OD) δ 1.88 (m, 6H, 3×CH_2), 2.67 (m, 6H, 3×CH_2), 2.90 (m, 2H, CH_2), 3.29 (m, 1H, CH), 3.67 (m, 1H, CH), 4.34 (m, 1H, CH), 6.84 (s, 1H, 5-H), 7.50 (s, 1H, 2-H); ESIMS *m*/*z* 365 (M + 1); analysis for $C_{16}H_{24}N_6O_4$ (364.4); calcd, C, 52.74; H, 6.64; N, 23.06; found, C, 54.03; H, 6.56; N, 23.02; $[\alpha]_D^{25}$ −63.3° (*c* 1.2, MeOH); R_f 0.30 (one spot).

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