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Synthesis, receptor binding, and activation studies of N(1)-alkyl-L-histidine containing thyrotropin-releasing hormone (TRH) analogues

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Abstract—Thyrotropin-releasing hormone (TRH) analogues in which the N(1)-position of the imidazole ring of the centrally placed histidine residue is substituted with various alkyl groups were synthesized and studied as agonists for TRH receptor subtype 1 (TRH-R1) and subtype 2 (TRH-R2). Analogue **3** (R = C_2H_5) exhibited binding affinity (K_i) of 0.012 μM to TRH-R1 that is about 1.1-fold higher than that of TRH. Several analogues were found to selectively activate TRH-R2 with greater potency than TRH-R1. The most selective agonist of the series **5** [R = $CH(CH_3)_2$] was found to activate TRH-R2 with a potency (EC_{50}) of 0.018 μM but could only activate TRH-R1 at EC_{50} value of 1.6 μM; that is, exhibited 88-fold greater potency for TRH-R2 versus TRH-R1. The results of this study indicate that modulation of central histidine residue is important for designing analogues which were selective agonist at TRH receptor subtypes.

1. Introduction

Thyrotropin-releasing hormone (TRH, L-pGlu-L-His-L-ProNH_2, **1**) (Fig. 1), a hypothalamic neuropeptide, has been manifested in a wide range of biological responses.[1–3] TRH functions as a neuroendocrine hormone by increasing secretion of thyrotropin (thyroid-stimulating hormone, TSH), leading to an elevation of thyroid hormone levels, and prolactin. Besides this hormonal activity, TRH has also been long recognized as a modulatory neuropeptide in the CNS and has been shown to exert a variety of extrahypothalamic effects in animals. At pharmacologic doses, TRH exhibits a plethora of non-endocrine actions ranging from analeptic, antidepressant, anticonvulsant, neurotransmitter, and neuromodulator action, to its complex interaction with cardiovascular and cerebrovascular system. Therefore, this peptide has been implicated as a promising lead compound for treating motor neuron diseases, spinal cord trauma, and Alzheimer's disease.[4,5] The use of TRH as a CNS-active agent is, however, hampered by several factors. For example, the TRH peptide has a short half-life, it does not effectively penetrate the blood–brain barrier (BBB), and its endocrine effect is usually manifested at doses causing significant cognitive improvement.[6]

TRH is a unique peptide, not only being orally active, but also as one to demonstrate its primary receptor binding site within the seven-transmembrane helical region of its G-protein coupled receptor (GPCR), which activates the phosphatidylinositol-calcium-protein kinase C transduction pathway after the interaction with

Abbreviations: pGlu, pyroglutamic acid; His, histidine; Im, imidazole; ProNH_2, prolinamide; DCC, 1,3-dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, 1,3-dicyclohexylurea; DIC, 1,3-diisopropylcarbodiimide; DIU, 1,3-diisopropylurea; DMF, *N*,*N*′-dimethylformamide; EDT, 1,2-ethanedithiol; EtOAc, ethyl acetate; HONB, *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide; MBHA, 4-methylbenzhydrylamine resin; MeOH, methanol; OTcp, trichlorophenyl; TBTU, 2-(1*H*-benztriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid.

Keywords: 1-Alkyl-L-histidine; TRH; TRH receptor subtype 1; TRH receptor subtype 2; Peptides.

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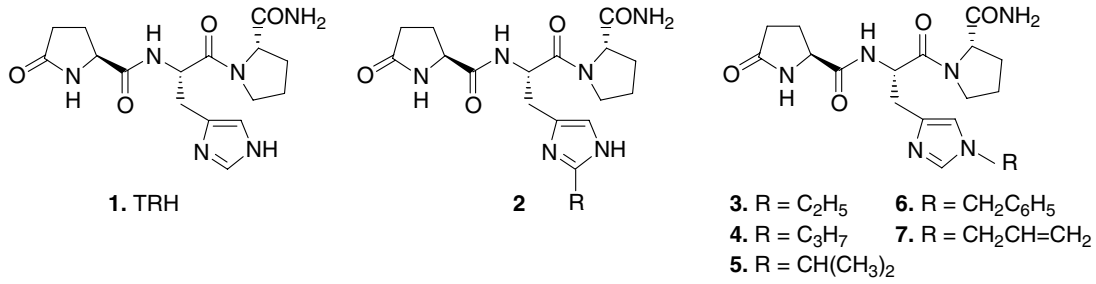


Figure 1. Structures of TRH (**1**), 2-alkyl-L-histidine-TRH analogues (**2**) and newly synthesized 1-alkyl-L-histidine-TRH analogues **3**–**7**.

TRH.[7] The first TRH receptor (TRH-R1) was cloned from a mouse pituitary tumor and several other orthologous receptors were cloned from different species, including rat, *Catostomus commersoni*, and human.[8–10] More recently, a second subtype of TRH receptor (TRH-R2) was identified in rats, mouse, and *C. commersoni*.[11–14] Amino acid sequences of the two subtypes of TRH receptor from the same species reveal a 51% overall similarity.[15] Subsequent comparison of TRH-R1 and TRH-R2 has been made regarding their tissue distribution. TRH-R1 is highly expressed in the anterior pituitary and is mainly involved in the signaling of TRH within neuroendocrine brain regions, the autonomic nervous system, and the visceral brainstem regions of rat. TRH-R1 exhibits only a very limited mRNA expression pattern in other regions of the CNS. In contrast, TRH-R2 is strongly expressed in rat brain and spinal cord, but is not detectable in the pituitary.[15] The specific expression of TRH-R2 in brain areas that are important for the transmission of somatosensory signals and higher CNS functions indicates that in the CNS this receptor subtype may be of major functional importance. Therefore, it has been proposed that TRH-R2 would possibly mediate biological responses of a therapeutically useful CNS selective TRH analogue; whereas, in contrast, TRH-R1 would mediate its TSH-releasing effects.

All three amino acid residues of TRH have been implicated in eliciting its physiological response(s), with pGlu and His residues accounting for almost all of TRH binding energy and affinity.[16] We have earlier investigated the role of pGlu residue in binding and the effect of bio-isosteric replacement of the carbonyl group, in tandem with methyl substitution on N(1)-position of imidazole ring in histidine residue, in the binding affinity of TRH to yield selective agonists.[17] Histidine often plays a critical role in the biological activities of many enzymes, peptide hormones, neuropeptides, and peptide antibiotics. The physicochemical properties of the side-chain heteroaromatic imidazole ring, viz. its acid–base characteristics, aromatic nature, hydrogen bond donor as well as acceptor properties, and ring tautomerism, make histidine a unique DNA encoded amino acid. Modulation of histidine either by substitution on the imidazole ring or by its replacement with other amino acids in bioactive peptides such as TRH can have profound influence on its biological activities. Recently, in one such study, we have observed that placement of a hydrophobic alkyl group at the C(2)-position of the imidazole ring of the centrally placed histidine residue resulted in a series of highly selective agonists **2** (Fig. 1) for TRH-R2 exhibiting no activation of TRH-R1.[18] Earlier it has been reported that only one analogue—one containing methyl substitution at N(1)-position of imidazole {[N(1)–Me–His]TRH} exhibited superior binding affinity and potency at TRH-R1 than the parent peptide.[19] These observations suggest that any change in the steric, aromatic and acid–base properties of the imidazole ring of the central histidine residue in TRH could lead to analogues which show selectivity to any of the two receptor subtypes. Furthermore, the high potency shown by [N(1)–Me–His]TRH (about 7-fold more potent to TRH) and the complete loss of activity in the tautomeric peptide in which methyl group is placed at the N(3)-position on the imidazole ring exemplified by [N(3)–Me–His]TRH[19] make N(1)-position all the more an important site for studying the effect of modification on receptor binding and receptor activation profile of TRH.

We report herein, synthesis of a series of N(1)-substituted L-histidine containing TRH analogues **3**–**7** (Fig. 1) and results of receptor binding and activation studies to determine the effect of modification at N(1)-position. We expected that if steric bulk at N(1)-position facilitates hydrophobic interactions, a currently held view in connection with increased activity of [N(1)–Me–His]TRH analogue of TRH, then placement of an ethyl, a propyl or an isopropyl group at the N(1)-position of histidine residue should provide analogues producing activity comparable to that of TRH, provided steric bulk of higher alkyl groups does not lead to overcrowding in the binding pocket. On the other hand, an allyl group will impart polar character owing to its double bond that may facilitate differentiating the role of steric versus electronic properties required in binding at the central histdine residue. In contrast, benzyl substitution at the N(1)-position of the imidazole ring may facilitate π–π stacking interactions at receptor binding pocket.

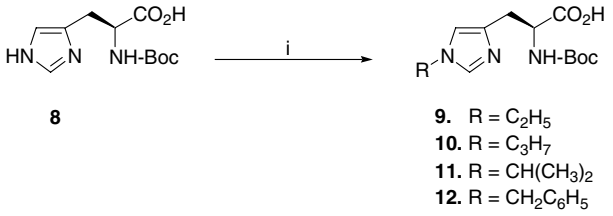
2. Results and discussion

2.1. Chemistry

Reaction of commercially available *N*-α-(*tert*-butoxycarbonyl)-L-histidine **8** with NaH at −15 °C for 30 min in anhydrous DMF, followed by addition of the appropriate commercially available alkyl halides at −5 °C for

4 h, readily produced *N*-α-(*tert*-butoxycarbonyl)-1-alkyl-L-histidines **9**–**12** (Scheme 1).[20] Unfortunately, all attempts toward incorporation of an allyl group using this methodology resulted in the recovery of starting material along with some unidentified higher molecular weight products. Therefore, allyl group at the N(1)-position of histidine was introduced using methodology described in Scheme 2.[21] Commercially available L-histidine methyl ester dihydrochloride (**13**) upon treatment with 1,1′-carbonyl-diimidazole in DMF at 60 °C for 5 h afforded (7*S*)-5,6,7,8-tetrahydro-7-(methoxycarbonyl)-5-oxoimidazo-[1,5-*c*]pyrimidine **14** as a crystalline solid in good yield. Allylation of the pyrimidine derivative **14** with allyl iodide in acetonitrile at 80 °C for 24 h afforded (7*S*)-2-allyl-5,6,7,8-tetrahydro-7-methoxycarbonyl-5-oxoimidazo[1,5-*c*]pyrimidinium iodide (**15**). The latter compound **15** upon ring opening with 6 N HCl solution at 100 °C for 24 h afforded 1-allyl-L-histidine (**16**) in excellent yields as its dihydrochloride salt. C-Protected amino acid, 1-allyl-L-histidine methyl ester (**17**), required for peptide synthesis was then obtained by passing a slow stream of anhydrous hydrogen chloride gas through a solution of compound **16** in anhydrous methyl alcohol for 45 min at 4 °C, and subsequent liberation of the free base by neutralization with a solution of 7 N NH_3 in CH_3OH for 30 min.

Synthesis of tripeptides, L-pGlu-(1-alkyl)-L-His-L-Pro-NH_2 **3**–**6**, was carried out on the solid support. All peptides were assembled using solid-phase peptide synthesis methodology on a CS BIO 136 fully automated peptide synthesizer. Briefly, 4-methylbenzhydrylamine-functionalized, 1% cross-linked polystyrene resin (MBHA resin, **18**) was neutralized with DIEA (10% solution in DCM), and subsequently coupled with Boc-L-proline by means of TBTU in DMF to produce protected amino acid-linked resin. Deprotection of *t*-Boc group with TFA (40% solution in DCM) followed by neutralization with DIEA (10% solution in DCM) provided free amino acid-linked resin **19**. This was subsequently submitted to further coupling and deprotection cycles with the protected amino acids **9**–**12** and finally with *Z*-L-pGlu-OH to afford peptide-resins **24**–**27**. The intermediate coupling steps were monitored by employing Kaiser's test, which showed negative results after each coupling step due to complete reaction of primary amino group. Simultaneous cleavage from the resin support of the tripeptides **24**–**27** and removal of side-chain protection group was affected using trifluoromethanesulfonic acid (TFMSA) in TFA in the presence of thioanisole and 1,2-ethanedithiol (EDT) as scavengers for 2 h at ambient temperature to afford peptide amides **3**–**6** (Scheme 3).



8

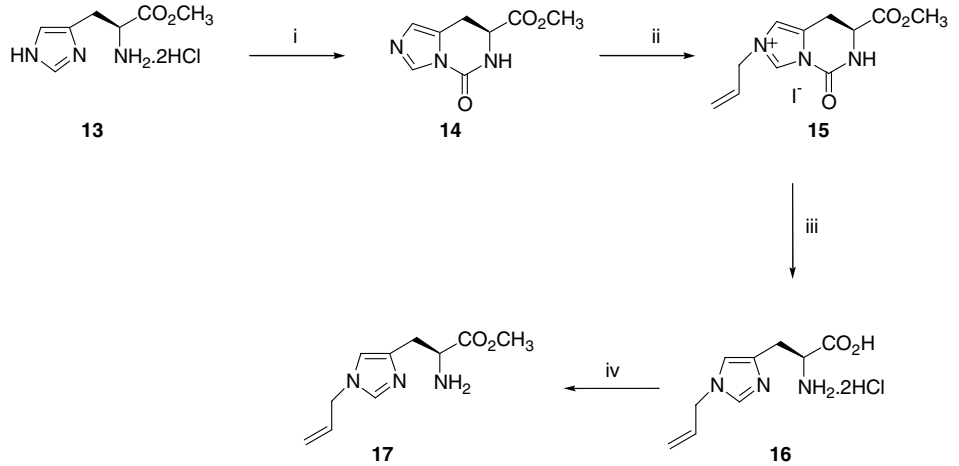
9. R = C_2H_5
10. R = C_3H_7
11. R = $CH(CH_3)_2$
12. R = $CH_2C_6H_5$

Scheme 1. Reagents and conditions: (i) NaH, RI, CH_3CN, −15 to −5 °C.

The 2,4,5-trichlorophenyl active ester (**28**) of L-pGlu upon reaction with 1-allyl-L-histidine methyl ester (**17**) at 4 °C for 36 h in anhydrous EtOAc gave dipeptide methyl ester **29** in good yield (Scheme 4). Basic hydrolysis of the latter dipeptide **29** with methanolic 0.5 N NaOH solution at 0 °C for 30 min followed by neutralization of the resultant peptide salt with Dowex ion-exchange resin (50 × 2–200, H^+ form) produced dipeptide carboxylic acid **30**. Coupling of the latter dipeptide **30** with commercially available L-prolinamide in the presence of DIC and HONB in anhydrous DMF at 4 °C for 36 h yielded required tripeptide **7** in moderate yield (Scheme 4).

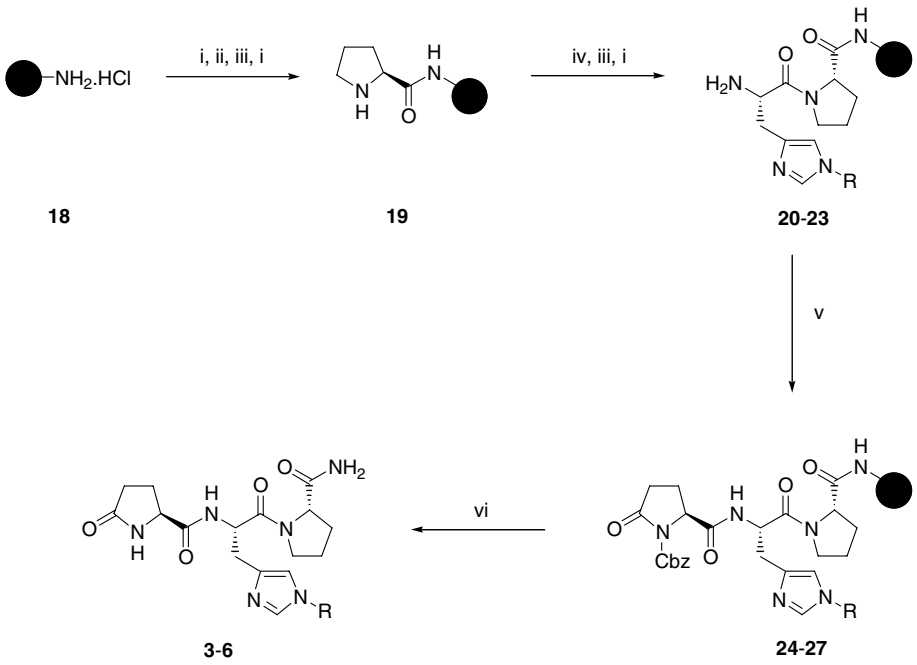
2.2. Pharmacology at TRH-R1 and TRH-R2

Synthesized TRH analogues **3**–**7** were examined for their affinity for TRH-R1 and TRH-R2 and their ability to serve as agonists for the receptors.[22] Affinities, reported as K_i (μM) values, were determined by measuring the

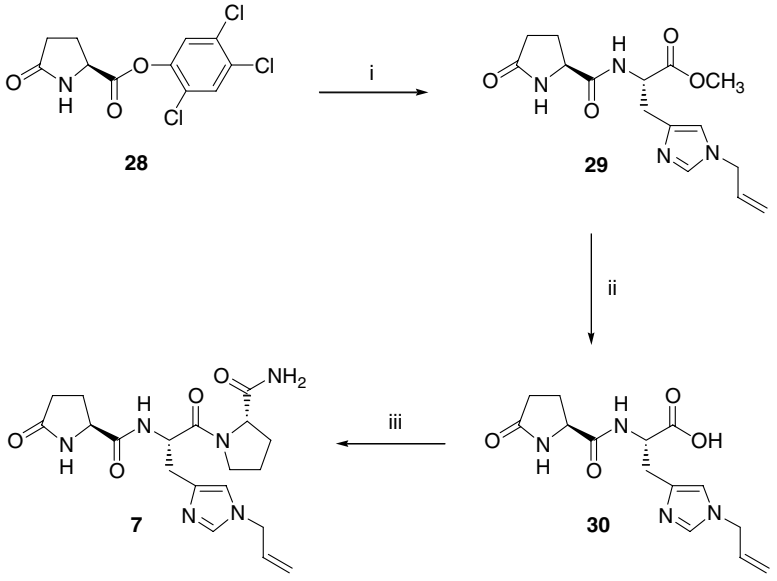


13 **14** **15** **17** **16**

Scheme 2. Reagents and conditions: (i) 1,1′-carbonyl-diimidazole, DMF, 60 °C, 5 h; (ii) $CH_2{=}CHCH_2I$, CH_3CN, 80 °C, 24 h; (iii) 6 N HCl, 100 °C, 24 h; (iv) a—HCl, MeOH, 4 °C, 45 min; b—7 N NH_3/CH_3OH, 30 min.



Scheme 3. Reagents and conditions: (i) 10% DIEA, DCM, 10 min; (ii) Boc-L-Pro-OH, TBTU, 10% DIEA, DMF, 90 min; (iii) 40% TFA, DCM, 20 min; (iv) Boc-(1-alkyl)-L-His-OH (**9**–**12**), TBTU, 10% DIEA, DMF, 90 min; (v) Cbz-L-pGlu-OH, TBTU, 10% DIEA, DMF, 90 min; (vi) TFMSA, EDT, thioanisole, TFA, 2 h, rt.

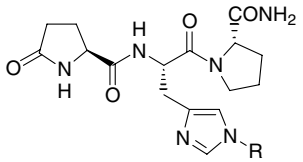


Scheme 4. Reagents and conditions: (i) 1-allyl-L-His-OMe (**17**), EtOAc, 4 °C, 36 h; (ii) 0.5 N NaOH, MeOH, 0 °C, 30 min, Dowex ion-exchange (50 × 2–200, H^+ form); (iii) L-ProNH_2, DIC, HONB, DMF, 4 °C, 36 h.

concentration of the analogue required to compete with 2 nM [^{3}H][N(1)–Me–His]TRH for binding to the receptor. [N(1)–Me–His]TRH is known to bind to TRH-R1 and TRH-R2 with affinities higher than TRH. The agonist behavior of the analogues was tested in HEK 293EM cells stably expressing TRH-R1 or TRH-R2 by incubating the cells with various doses of the analogues as described.[23,24] The extent of agonist behavior was then determined by measuring signaling through a reporter gene and the data are reported as EC_{50} (μM) values (Table 1).

Four of the synthesized peptides **4**–**7** exhibited modest binding affinity (K_i) to TRH-R1 and TRH-R2, but all were lower than TRH. Analogue **3** (R = C_2H_5) exhibited high binding affinity (K_i = 0.012 μM and K_i = 0.037 μM) compared to TRH (K_i = 0.02 μM and K_i = 0.01 μM) at TRH-R1 and TRH-R2, respectively. Thus, analogue **3** displayed binding affinity that is about 1.1-fold higher than that of TRH at TRH-R1, and 3.7-fold lower than that to TRH at TRH-R2. The order of affinities to both receptors was: analogue **3** (R = C_2H_5) > **5**

Table 1. Binding affinities K_i (μM) and signaling (activation) potencies EC_{50} (μM) produced by TRH analogues **3**–**7** for TRH-R1 and TRH-R2



Compound	R	K_i[a] (μM)		EC_{50}[b] (μM)		
		TRH-R1	TRH-R2	TRH-R1	TRH-R2	Fold selectivity (TRH-R2)
3	CH_2CH_3	0.012 (0.010–0.015)	0.037 (0.033–0.040)	0.016 (0.0077–0.032)	0.0033 (0.0012–0.0094)	5
4	$CH_2CH_2CH_3$	1.1 (0.91–1.3)	3.8 (2.6–5.4)	0.58 (0.37–0.91)	0.12 (0.066–0.22)	5
5	$CH(CH_3)_2$	1.8 (1.4–2.4)	0.69 (0.43–1.1)	1.6 (1.1–2.2)	0.018 (0.0075–0.040)	88
6	$CH_2C_6H_5$	4.5 (3.0–6.7)	11 (3.3–36)	0.96 (0.72–1.3)	0.26 (0.18–0.36)	4
7	$CH_2CH{=}CH_2$	9.9 (4.9–20)	24 (7.2–80)	5.6 (3.8–8.2)	0.38 (0.25–0.59)	15
TRH	H	0.02 (0.01–0.03)	0.01 (0.008–0.015)	0.003 (0.002–0.004)	0.003 (0.002–0.007)	—
[N(1)–Me–His]TRH		0.003 (0.002–0.005)	0.0021 (0.002–0.005)	0.0005 (0.0004–0.0008)	0.0002 (0.0002–0.0005)	—

All data are means (95% confidence limits) of nine doses of analogues assayed in duplicate determinations in three experiments.

[a] For binding, cells expressing TRH-R1 or TRH-R2 were incubated with 1 nM [^{3}H][N(1)–Me–His]TRH in the absence or presence of various doses of unlabeled TRH analogues for 1 h at 37 °C.

[b] For signaling, cells expressing TRH-R1 or TRH-R2 and a CREB-luciferase reporter were incubated with various doses of TRH analogues for 6 h at 37 °C, and luciferase activity was measured. Experiments were performed with intact HEK293EM cells.

[R = $CH(CH_3)_2$] > **4** (R = $CH_2CH_2CH_3$) > **6** (R = $CH_2C_6H_5$) > **7** (R = $CH_2CH{=}CH_2$). More importantly, in addition to a distinct hierarchy, there was definite preference as an agonist of the analogues for TRH-R2 over TRH-R1—analogue **5** [R = $CH(CH_3)_2$] > **7** (R = $CH_2CH{=}CH_2$) > **3** (R = C_2H_5) = **4** (R = $CH_2CH_2CH_3$) = **6** (R = $CH_2C_6H_5$). Of note, placement of an isopropyl group at N(1)-position led to highly promising and most selective analogue **5** [R = $CH(CH_3)_2$] that exhibited 88-fold selectivity to TRH-R2 versus TRH-R1, while displaying full agonist activity at TRH-R2 with high potency (EC_{50} = 0.018 μM). Similarly, placement of an ethyl group at N(1)-position led to most potent analogue **3** (R = C_2H_5) that exhibited high agonist potency [EC_{50} = 0.0033 μM] comparable to TRH [EC_{50} = 0.003 μM] with 5-fold selectivity to TRH-R2 versus TRH-R1.

3. Conclusions

Important research tools could be obtained by the discovery of TRH peptides that selectively and potently bind and activate the TRH receptor subtypes 1 and 2. These modified peptide analogues could serve as model template to study TRH receptors within the CNS. In the present study, we have attempted to determine the effects of TRH peptides synthesized through modification at the N(1)-position in the imidazole ring of the histidine residue on receptor subtype specificity. We found two analogues produced selectivity for mouse TRH-R1 and TRH-R2 at low micro-molar concentrations. In particular, analogue **5** was found to activate TRH-R2 with a potency of 0.018 μM but could only activate TRH-R1 at 1.6 μM (88-fold selectivity). Similarly, analogue **3** binds to TRH-R1 with an affinity of 0.012 μM that is about 1.1-fold higher than that of TRH. These observations indicate that binding affinity and activation potency of reported TRH peptides for TRH-R1 and TRH-R2 was critically dependent on the modulation of histidine residue. In conclusion, these newly synthesized receptor subtype specific TRH analogues hold promise as agonist for both TRH receptor subtypes.

4. Experimental

4.1. Synthesis

Compounds were routinely checked for their purity on pre-coated silica gel G_{254} TLC plates (Merck) and the spots were visualized under UV spectrophotometer and then by exposing them to iodine vapors. Column chromatographic purification was carried out on Merck silica gel (230–400 mesh). ^{1}H NMR spectra were recorded on 300 MHz Bruker FT–NMR (Avance DPX 300) spectrometer using tetramethylsilane (TMS) as internal standard and the chemical shifts are reported in δ units. The sample concentration in each case was approximately 7 mg in 0.5 mL of solvent. Mass spectra were recorded on HRMS (Finnigan Mat LCQ spectrometer) (APCI/ESI). Elemental analyses were recorded on Elementar Vario EL spectrometer. Optical rotations were recorded on a Perkin-Elmer 241MC polarimeter. All final peptides **3**–**7** were also checked for their homogeneity on a Shimadzu LS10AT HPLC system using a Merck Lichrospher® 100 RP-18 (10 μm) column. The samples were analyzed using a solvent system of CH_3CN/H_2O/TFA (95:5:0.05%) at a flow rate of 1 mL/min.

4.2. General method for the synthesis of *N*-α-(*tert*-butoxycarbonyl)-1-alkyl-L-histidines (9–12)

Sodium hydride (60% suspension, 11.7 mmol) was placed in a two-necked flask, washed with hexanes (2× 10 mL), and dried under vacuum. *N*-α-(*tert*-Butoxycarbonyl)-L-histidine (**8**, 3.9 mmol) in CH_3CN (20 mL)

was added under a nitrogen atmosphere at −15 °C. The reaction mixture was stirred for another 30 min at −15 °C, and then alkyl iodide (7.8 mmol) was added. The temperature of the reaction was raised to −5 °C, and the reaction was stirred for another 4 h under N_2. The reaction was quenched by addition of methanol (5 mL) and residue was extracted with chloroform (4× 50 mL), and the combined organic layers were dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by silica gel (230–400 mesh) column chromatography eluting with 13% CH_3OH in CH_2Cl_2 to afford *N*-α-(*tert*-butoxycarbonyl)-1-alkyl-L-histidines **9**–**12** in good yields.

4.2.1. *N*-α-(*tert*-Butoxycarbonyl)-1-ethyl-L-histidine (9). Yield: 79%; oil, ^{1}H NMR (CD_3OD): δ 7.96 (s, 1H, 2-Ar-H), 6.94 (s, 1H, 5-Ar-H), 4.02 (m, 1H, CH), 3.95 (q, 2H, N–CH_2, J = 7.3 Hz), 3.03 (m, 2H, CH_2), 1.42 (t, 3H, CH_3, J = 7.3 Hz), 1.38 (s, 9H, 3× CH_3); MS (APCI): *m*/*z* 284 (M+1); Anal. Calcd for $C_{13}H_{21}N_3O_4$ (283.2): C, 55.11; H, 7.47; N, 14.83. Found: C, 54.79; H, 7.09; N, 14.67; $[\alpha]_D^{25}$ +15.4° (*c* 1, CH_3OH).

4.2.2. *N*-α-(*tert*-Butoxycarbonyl)-1-propyl-L-histidine (10). Yield: 75%; oil, ^{1}H NMR (CD_3OD): δ 7.98 (s, 1H, 2-Ar-H), 6.90 (s, 1H, 5-Ar-H), 4.10 (m, 1H, CH), 3.91 (t, 2H, N–CH_2, J = 7.0 Hz), 3.07 (m, 2H, CH_2), 1.78 (m, 2H, CH_2), 1.39 (s, 9H, 3× CH_3), 0.86 (t, 3H, CH_3, J = 7.3 Hz); MS (APCI): *m*/*z* 298 (M+1); Anal. Calcd for $C_{14}H_{23}N_3O_4$ (297.4): C, 56.55; H, 7.80; N, 14.13. Found: C, 56.34; H, 7.99; N, 14.51; $[\alpha]_D^{25}$ +13.5° (*c* 1, CH_3OH).

4.2.3. *N*-α-(*tert*-Butoxycarbonyl)-1-isopropyl-L-histidine (11). Yield: 71%; oil; ^{1}H NMR (CD_3OD): δ 8.02 (s, 1H, 2-Ar-H), 6.80 (s, 1H, 5-Ar-H), 4.10 (m, 1H, CH), 4.06 (m, 1H, N–CH), 2.96 (m, 2H, CH_2), 1.58 (d, 6H, 2× CH_3, J = 6.6 Hz), 1.43 (s, 9H, 3× CH_3); MS (APCI): *m*/*z* 298 (M+1); Anal. Calcd for $C_{14}H_{23}N_3O_4$ (297.4): C, 56.55; H, 7.80; N, 14.13. Found: C, 56.87; H, 7.65; N, 13.88; $[\alpha]_D^{25}$ +17.6° (*c* 1, CH_3OH).

4.2.4. *N*-α-(*tert*-Butoxycarbonyl)-1-benzyl-L-histidine (12). Yield: 25%; oil; ^{1}H NMR (CD_3OD): δ 7.56 (s, 1H, 2-Ar-H), 7.14 (s, 5H, benzyl), 6.92 (s, 1H, 5-Ar-H), 5.23 (s, 2H, N–CH_2), 4.05 (m, 1H, CH), 2.96 (m, 2H, CH_2); MS (APCI): *m*/*z* 346 (M+1); Anal. Calcd for $C_{18}H_{23}N_3O_4$ (345.2): C, 62.59; H, 6.71; N, 12.17. Found: C, 62.88; H, 7.12; N, 12.45; $[\alpha]_D^{25}$ +19.6° (*c* 1, CH_3OH).

4.3. Synthesis of 1-allyl-L-histidine methyl ester (17)

To a solution of 1-allyl-L-histidine·2HCl[21] (**16**, 100 mmol) in anhydrous methanol (100 mL) cooled to 0 °C was bubbled dry hydrogen chloride gas for 45 min. The solution was allowed to stand overnight at ambient temperature. The solvent was evaporated under reduced pressure to afford 1-allyl-L-histidine methyl ester dihydrochloride. A 7 N solution of ammonia in CH_3OH (10 mL) was added to the dihydrochloride salt, and the reaction mixture was allowed to stand for 30 min at ambient temperature. Evaporation of solvent under reduced pressure and subsequent extraction of the resulting residue with $CHCl_3$ (3× 15 mL) afforded 1-allyl-L-histidine methyl ester **17**.

Yield: 91%; oil; ^{1}H NMR ($CDCl_3$): δ 7.63 (s, 1H, 2-Ar-H), 6.98 (s, 1H, 5-Ar-H), 5.99 (m, 1H, CH), 5.17 (m, 2H, CH_2), 4.58 (d, 2H, N–CH_2, J = 5.1 Hz), 4.04 (m, 1H, CH), 3.66 (s, 3H, OCH_3), 3.01 (m, 2H, CH_2); MS (ESI): *m*/*z* 210 (M+1); Anal. Calcd for $C_{10}H_{15}N_3O_2$ (209.3): C, 57.40; H, 7.23; N, 20.08. Found: C, 57.71; H, 7.03; N, 20.41; $[\alpha]_D^{25}$ −17.5° (*c* 1, CH_3OH).

4.4. General method for the synthesis of (2*S*)-1-{(2*S*)-3-(1-alkyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarboxamido]propanoyl}azolane-2-carboxamides [L-pGlu-(1-alkyl)-L-His-L-Pro-NH_2] (3–6)

Solid-phase peptide synthesis of TRH analogues was performed on a fully automated CS Bio 136 peptide synthesizer. 4-Methylbenzhydrylamine (MBHA·HCl) resin (**18**, 500 mg, 0.31 mmol) was charged into the reaction vessel and the requisite amino acids (0.93 mmol) were loaded sequentially into the amino acid vessels. The MBHA·HCl resin **18** was neutralized with DIEA (10% in DCM) for 5 min and then washed with DMF (2× 10 mL) and once with DCM (10 mL). The coupling of the first amino acid Boc-L-Pro-OH was done in the presence of coupling reagent TBTU and DIEA (10% in DCM) for 90 min. After the completion of coupling step, the *t*-Boc group was cleaved with trifluoroacetic acid (40% solution in DCM) for 20 min. The amino acid-linked resin was again neutralized with DIEA (10% in DCM) for 5 min and washed with DMF (2× 10 mL) and once with DCM (10 mL) to afford **19**. The coupling and deprotection steps were repeated to obtain desired peptide resins **24**–**27**. All coupling reactions were monitored by Kaiser's test for completion. After the successful completion of synthesis, the peptides were cleaved from solid support using TFMSA (trifluoromethanesulfonic acid) protocol. Accordingly, dry peptide-linked resins **24**–**27** were taken in two-necked round-bottomed flask equipped with a drying tube and a rubber septum. 1,2-Ethanedithiol (0.5 mL), thioanisole (1.0 mL), and trifluoroacetic acid (10 mL) were added to the reaction vessel through septum and reaction mixture was stirred for 10 min at 4 °C. Trifluoromethanesulfonic acid (1.0 mL) was then added and reaction mixture was stirred at ambient temperature for 2 h. The crude peptide was separated from solid support by filtration and resin was washed with TFA (3× 4 mL). Solvent was removed under reduced pressure and residue was neutralized with saturated ammonium bicarbonate solution. The non-polar impurities were removed by extracting the aqueous layer with diethyl ether (3× 10 mL). The aqueous layer was evaporated under reduced pressure to afford crude peptide, which upon purification using column chromatography over neutral alumina using $CHCl_3$/CH_3OH (4:1) as eluant produced tripeptides **3**–**6** in high purity.

4.4.1. (2*S*)-1-{(2*S*)-3-(1-Ethyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarboxamido]propanoyl}azolane-2-carboxamide [L-pGlu-(1-ethyl)-L-His-L-Pro-NH_2] (3). Mp 100–101 °C (dec); ^{1}H NMR (CD_3OD): δ 8.33 (s, 1H,

2-Ar-H), 7.34 (s, 1H, 5-Ar-H), 4.84 (m, 1H, α-CH), 4.52 (m, 1H, α-CH), 4.43 (m, 1H, α-CH), 4.22 (q, 2H, N–CH_2, J = 7.1 Hz), 3.64 (m, 2H, CH_2), 3.08 (m, 2H, Im-CH_2), 2.40–1.28 (m, 8H, 4× CH_2), 0.89 (t, 3H, CH_3, J = 7.2 Hz); MS (ESI): m/z 391 (M+1); Anal. Calcd for $C_{18}H_{26}N_6O_4$ (390.2): C, 55.37; H, 6.71; N, 21.52. Found: C, 55.45; H, 6.92; N, 21.73; R_f = 0.57 [CH_3OH/25% NH_4OH/$CHCl_3$ (20:2:78)]; HPLC: t_R = 5.74 min, purity: 98.2%.

4.4.2. (2*S*)-1-{(2*S*)-3-(1-Propyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarboxamido]propanoyl}azolane-2-carboxamide [L-pGlu-(1-propyl)-L-His-L-Pro-NH_2] (4). Mp 97–98 °C (dec); ^{1}H NMR (CD_3OD): δ 8.74 (s, 1H, 2-Ar-H), 7.46 (s, 1H, 5-Ar-H), 4.94 (m, 1H, α-CH), 4.46 (m, 1H, α-CH), 4.13 (m, 2H, N–CH_2,), 3.75 (m, 1H, α-CH), 3.29 (m, 2H, Im-CH_2), 2.30 (m, 2H, CH_2), 2.04–1.86 (m, 6H, 3× CH_2), 1.17 (t, 3H, CH_3, J = 7.1 Hz), 0.97 (m, 4H, 2× CH_2); MS (ESI): m/z 405 (M+1); Anal. Calcd for $C_{19}H_{28}N_6O_4$ (404.2): C, 56.42; H, 6.98; N, 20.78. Found: C, 56.09; H, 6.78; N, 21.11; R_f = 0.60 [CH_3OH/25% NH_4OH/$CHCl_3$ (20:2:78)]; HPLC: t_R = 5.97 min, purity: 97.6%.

4.4.3. (2*S*)-1-{(2*S*)-3-(1-Isopropyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarboxamido]propanoyl}azolane-2-carboxamide [L-pGlu-(1-isopropyl)-L-His-L-Pro-NH_2] (5). Mp 93–94 °C (dec); ^{1}H NMR (CD_3OD): δ 8.66 (s, 1H, 2-Ar-H), 7.51 (s, 1H, 5-Ar-H), 4.57 (m, 1H, α-CH), 4.44 (m, 1H, α-CH), 4.21 (m, 1H, α-CH), 3.76 (m, 2H, Im-CH_2), 3.11 (m, 1H, N–CH), 2.43 (m, 2H, CH_2), 1.99 (m, 2H, CH_2), 1.52 (d, 6H, 2× CH_3, J = 5.3 Hz), 1.28–0.85 (m, 6H, 3× CH_2); MS (ESI): m/z 405 (M+1); Anal. Calcd for $C_{19}H_{28}N_6O_4$ (404.2): C, 56.42; H, 6.98; N, 20.78. Found: C, 56.84; H, 6.75; N, 20.99; R_f = 0.60 [CH_3OH/25% NH_4OH/$CHCl_3$ (20:2:78)]; HPLC: t_R = 5.88 min, purity: 98.0%.

4.4.4. (2*S*)-1-{(2*S*)-3-(1-Benzyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarboxamido]propanoyl}azolane-2-carboxamide [L-pGlu-(1-benzyl)-L-His-L-Pro-NH_2] (6). Mp 121–122 °C (dec); ^{1}H NMR (CD_3OD): δ 7.76 (s, 1H, 2-Ar-H), 7.40 (s, 5H, C_6H_5), 7.12 (s, 1H, 5-Ar-H), 5.35 (s, 2H, CH_2), 4.82 (m, 1H, α-CH), 4.24 (m, 1H, α-CH), 3.97 (m, 1H, α-CH), 2.97 (m, 2H, Im-CH_2), 2.13–1.66 (m, 10H, 5× CH_2); MS (ESI): m/z 453 (M+1); Anal. Calcd for $C_{23}H_{28}N_6O_4$ (452.2): C, 61.05; H, 6.24; N, 18.57. Found: C, 61.41; H, 6.55; N, 18.23; R_f = 0.62 [CH_3OH/25% NH_4OH/$CHCl_3$ (20:2:78)]; HPLC: t_R = 5.27 min, purity: 97.0%.

4.5. Synthesis of methyl (2*S*)-3-(1-allyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarbox-amido]propanoate [L-pGlu-(1-allyl)-L-His-OMe] (29)

L-pGlu-OTcp (**28**, 1 mmol) was added dropwise to 1-allyl-L-histidine methyl ester (**17**, 1 mmol) in EtOAc (50 mL) and reaction mixture was stirred at 4 °C for 36 h. Complete removal of the solvent under reduced pressure gave crude product. Column chromatographic purification on silica gel (230–400 mesh) using EtOAc–CH_3OH (94:6) as eluant provided dipeptide methyl ester **29**.

Yield: 62%; mp 95–96 °C; ^{1}H NMR (CD_3OD): δ 7.86 (s, 1H, 2-Ar-H), 7.14 (s, 1H, 5-Ar-H), 6.17 (m, 1H, CH), 5.44 (m, 2H, CH_2), 4.81 (m, 2H, N–CH_2), 4.41 (m, 1H, CH), 4.29 (m, 1H, CH), 3.91 (s, 3H, OCH_3), 3.24 (m, 2H, Im-CH_2), 2.64 (m, 2H, CH_2), 2.48 (m, 2H, CH_2); MS (ESI): m/z 321 (M+1); Anal. Calcd for $C_{15}H_{20}N_4O_4$ (320.3): C, 56.24; H, 6.29; N, 17.49. Found: C, 56.55; H, 6.09; N, 17.33.

4.6. Synthesis of (2*S*)-3-(1-allyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarboxamido]propanoic acid [L-pGlu-(1-allyl)-L-His-OH] (30)

L-pGlu-(1-allyl)-L-His-OMe (**29**, 1 mmol) was dissolved in a mixture of CH_3OH (50 mL) and 0.5 N NaOH (40 mL). The solution was stirred at 0 °C for 30 min, and after that 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-exchange resin. The resin was removed by filtration, and the filtrate was evaporated under reduced pressure to afford L-pGlu-(1-allyl)-L-His-OH **30**.

Yield: 63%; mp 132–134 °C (dec); ^{1}H NMR (CD_3OD): δ 7.35 (s, 1H, 2-Ar-H), 6.88 (s, 1H, 5-Ar-H), 6.00 (m, 1H, CH_2), 5.38 (m, 2H, CH_2), 4.71 (m, 2H, N–CH_2), 4.58 (m, 1H, α-CH), 4.15 (m, 1H, α-CH), 2.98 (m, 2H, Im-CH_2), 2.64 (m, 2H, CH_2), 2.48 (m, 2H, CH_2); MS (ESI): m/z 307 (M+1); Anal. Calcd for $C_{14}H_{18}N_4O_4$ (306.3): C, 54.89; H, 5.92; N, 18.29. Found: C, 55.15; H, 5.77; N, 17.95.

4.7. Synthesis of (2*S*)-1-{(2*S*)-3-(1-allyl-1*H*-4-imidazolyl)-2-[(2*S*)-5-oxoazolan-2-ylcarboxamido]propanoyl}azolane-2-carboxamide [L-pGlu-(1-allyl)-L-His-L-Pro-NH_2] (7)

Dipeptide acid (**30**, 1 mmol) was dissolved in DMF (25 mL), and HONB (1 mmol) was added to the solution followed by addition of DIC (1 mmol) under cooling at 4 °C. Stirring of the reaction mixture continued for another 5 min at 4 °C. L-ProNH_2 was then added in one portion. The reaction mixture was stirred for additional 36 h at 4 °C. Solvent evaporated under reduced pressure to afford crude product. Flash column chromatography using $CHCl_3$/CH_3OH (4:1) as the solvent system gave TRH analogue **7**.

Yield: 30%; mp 112-114 °C (dec); ^{1}H NMR (CD_3OD): δ 7.58 (s, 1H, 2-Ar-H), 6.99 (s, 1H, 5-Ar-H), 6.91 (br s, 1H, NH), 5.98 (m, 1H, CH), 5.21 (m, 2H, CH_2), 4.61 (m, 2H, N–CH_2), 4.55 (m, 1H, α-CH), 4.39 (m, 1H, α-CH), 4.18 (m, 1H, α-CH), 3.74 (m, 2H, Im-CH_2), 2.95 (m, 2H, CH_2), 2.29 (m, 4H, 2× CH_2), 1.96 (m, 4H, 2× CH_2); MS (ESI): m/z 403 (M+1); Anal. Calcd for $C_{19}H_{26}N_6O_4$ (402.2): C, 57.68; H, 6.78; N, 20.18. Found: C, 57.61; H, 6.59; N, 20.31; R_f = 0.55 [CH_3OH/25% NH_4OH/$CHCl_3$ (20:2:78)]; HPLC: t_R = 6.15 min, purity: 98.0%.

4.8. Receptor binding assay

HEK293EM cells stably expressing either TRH-R1 or TRH-R2 were grown in Dulbecco's modified Eagle's

medium containing 10% fetal bovine serum and 200 mg/mL hygromycin. For equilibrium binding experiments, HEK293EM cells stably expressing TRH–R1 or TRH–R2 were seeded into 24-well plates (1.5×10^5/well). After 48 h, cells were incubated at 37 °C for 1 h with [^{3}H][N(1)–Me–His]TRH (MeTRH, 2 nM) in Hanks' balanced salt solution, pH 7.4, and various doses of TRH analogues. Apparent inhibitory constants (K_i) were derived from curves fitted by non-linear regression analysis and drawn with the PRISM program 3 (GraphPadSoftware, Inc.) using the formula $K_i = (IC_{50})/(1 + ([L]/K_d))$ where IC_{50} is the concentration of unlabeled analogue that half-competes and K_d is the equilibrium dissociation constant for [^{3}H][N(1)–Me–His]TRH.

4.9. Assay of luciferase activity

On the day prior to transfection, the cells stably expressing either TRH-R1 or TRH-R2 were seeded into 24-well plates (1.5×10^5/well). After 16 h, the mediums were aspirated and the cells (approx 50% confluent) were co-transfected with plasmid DNA encoding CREB and CREB-activated luciferase gene (PathDetect CREB trans-Reporting System™, Stratagene) using the calcium phosphate method. On the second day, 6 h before the assay, medium containing 10% FBS was changed to medium containing 1% FBS, various concentrations of TRH and TRH analogues were added to the medium. Luciferase activity was measured 24 h after transfection. Cells were washed with phosphate-buffered saline and lysed with 0.2 mL lysis buffer (25 mM Gly-Gly, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, and 1% Triton X-100). Cell lysates (0.025 mL) were combined automatically with 0.125 mL reaction buffer (25 mM Gly-Gly, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol,15 mM KH_2PO_4, and 2 mM ATP) and 0.025 mL luciferin (0.4 mM) in reaction buffer and the luminescence measured for 10 s in a TR717 Microplate Luminometer (Tropix, Bedford, MA). The levels of luciferase activity detected by this assay reflect the activation of signaling by TRH.

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