

DOI: 10.1002/cmdc.201000481

Synthesis, Receptor Binding, and CNS Pharmacological Studies of New Thyrotropin-Releasing Hormone (TRH) Analogues

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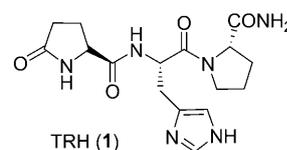
As part of our search for selective and CNS-active thyrotropin-releasing hormone (TRH) analogues, we synthesized a set of 44 new analogues in which His and pGlu residues were modified or replaced. The analogues were evaluated as agonists at TRH-R1 and TRH-R2 in cells in vitro, and in vivo in mice for analeptic and anticonvulsant activities. Several analogues bound to TRH-R1 and TRH-R2 with good to moderate affinities, and are full agonists at both receptor subtypes. Specifically, analogue **21 a** (R=CH₃) exhibited binding affinities (K_i values) of 0.17 μM for TRH-R1 and 0.016 μM for TRH-R2; it is 10-fold less potent than TRH in binding to TRH-R1 and equipotent with TRH in binding to TRH-R2. Compound **21 a**, the most selective agonist, activated TRH-R2 with a potency (EC_{50} value) of 0.0021 μM , but acti-

ated TRH-R1 at EC_{50} = 0.05 μM , and exhibited 24-fold selectivity for TRH-R2 over TRH-R1. The newly synthesized TRH analogues were also evaluated in vivo to assess their potencies in antagonism of barbiturate-induced sleeping time, and several analogues displayed potent analeptic activity. Specifically, analogues **21 a,b** and **22 a,b** decreased sleeping time by nearly 50% more than TRH. These analogues also displayed potent anticonvulsant activity and provided significant protection against PTZ-induced seizures, but failed to provide any protection in MES-induced seizures at 10 $\mu\text{mol kg}^{-1}$. The results of this study provide evidence that TRH analogues that show selectivity for TRH-R2 over TRH-R1 possess potent CNS activity.

Introduction

Thyrotropin-releasing hormone (TRH, L-pGlu-L-His-L-ProNH₂, **1**) is generated from a large precursor protein that contains multiple repeats of the TRH progenitor tetrapeptide Gln-His-Pro-Gly, and is the first hypothalamic hypophysiotropic neuropeptide to have had its sequence chemically elucidated.^[1–5] TRH plays a central role in the endocrine system and regulates several neurobiological activities.^[6] It exhibits antidepressant, analeptic, and neuroprotective effects and is considered a promising lead for the discovery of analogues in the treatment of epilepsy, motor neuron diseases, spinal cord trauma, and Alzheimer's disease.^[7–9] The rhodopsin/ β -adrenergic receptor subfamily of G-protein-coupled TRH receptors (TRH-R) in brain are ubiquitously distributed and found at highest densities in limbic structures, especially the amygdala and hypothalamus and in lower densities in brain stem and cerebellum.^[10,11] At present, two TRH receptor subtypes (TRH-R1 and TRH-R2) have been found in several species and exhibit an overall identity of ~50%.^[12–19] Studies conducted earlier demonstrated that TRH-R1 is highly expressed in the anterior pituitary, the neuroendocrine brain regions, the autonomic nervous system, and the visceral brain stem regions. TRH-R2 is expressed in the cerebellar cortex, mesencephalon, thalamic and subthalamic regions, and the brain stem.^[17,19] It has therefore been hypothesized that TRH-R2 could mediate some central nervous system (CNS) effects of TRH, whereas TRH-R1 mediates its thyroid-stimulating hormone (TSH)-releasing effects.

All three amino acid residues of TRH play a role in eliciting its physiological response(s), with pGlu and His residues ac-



counting for almost all of TRH binding energy and affinity. In our previous studies, several modified amino acids or carboxylic acid derived scaffolds were incorporated independently or simultaneously into the sequence of TRH at pGlu and His to

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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cmdc.201000481>; detailed experimental procedures and spectral data for intermediates **12 a–m**, **13 a–m**, **18 a–e**, and **19 a–e**.

synthesize a library of analogues.^[20–27] The results of these studies showed that TRH analogues in which His is modified at the N1(τ) or C2 positions of the imidazole ring with various bulky hydrophobic groups exhibit selectivity for TRH-R2. The TRH analogues in which pGlu is replaced with (1*R*)- or (1*S*)-3-oxo-1-cyclopentanecarboxylic acid (Ocp), and His is substituted at the C2 position, bind with high affinity (*K*) to both TRH-R1 and TRH-R2, whereas TRH analogues in which pGlu is replaced with L-pyro-2-aminoadipic acid (pAad), and His is substituted at the N1(τ) or C2 positions, exhibit good potency and selective activation of TRH-R2 over TRH-R1. These studies indicate that independent or simultaneous change of pGlu and His residues in TRH alters its receptor binding affinity and signal transducing potency.

In continuation, herein we report the synthesis of four new series of TRH analogues that involve independent or simultaneous modifications at the pGlu and His residues of the parent peptide in order to gain additional insight into the structure–activity relationships and biological activities (Figure 1). These

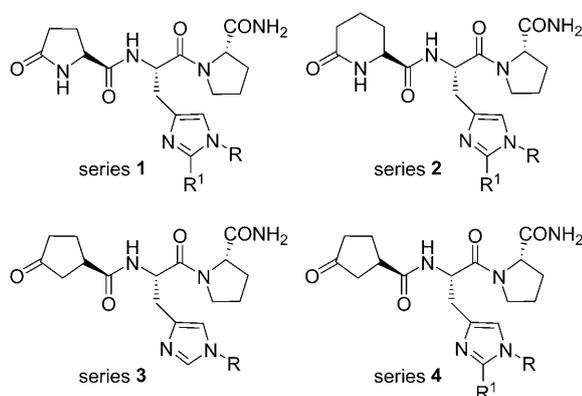


Figure 1. General structures of the four series of synthesized TRH analogues.

new series allowed a comparison of carboxylic acids at the N-terminal residue and the effects of substitution at the His residue on the binding affinity and potency of the modified peptidomimetics. The His residue was replaced with either N1(τ) substituted or 1,2-disubstituted-L-His, whereas the pGlu residue was replaced with (1*R*)-3-Ocp or L-pAad. In addition to their receptor binding studies, the synthesized TRH analogues were also evaluated *in vivo* in mice for CNS activity on the antagonism of pentobarbital-induced sleep duration as a primary screen. The most promising analogues were then evaluated in the selected models of epilepsy to assess their promise as future drugs in the treatment of epilepsy.

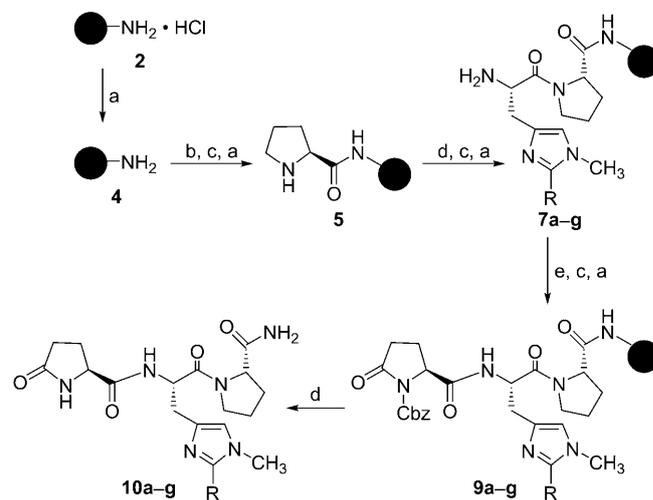
Results and Discussion

Chemistry

Boc-1,2-dialkyl-L-His-OH compounds **6a–m** were prepared from commercially available L-His-OMe using a multistep synthetic procedure as discussed previously.^[28–30] L-pAad-OPfp (**15**) was synthesized from commercially available L-Lys(Z)-OH in

four steps as described earlier.^[31] Boc-1-alkyl-L-His-OH compounds **17a–e** were synthesized from Boc-L-His-OH in one step as reported.^[32] (1*R*)-3-Ocp-OH (**20**) was synthesized from 4-vinylcyclohexene in four steps and chemically resolved using a well-established procedure.^[33–35]

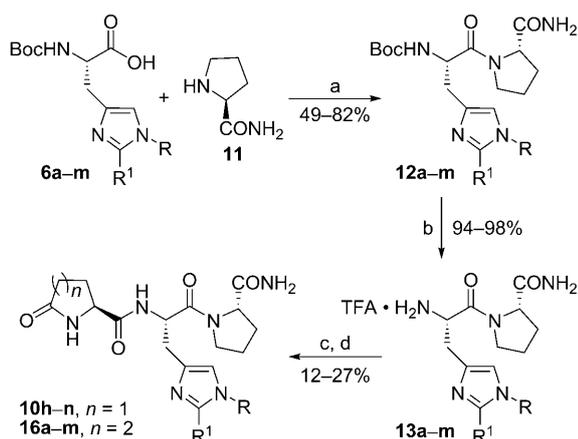
A solid-phase peptide synthesis protocol was used for the synthesis of targeted tripeptides **10a–g** (Scheme 1). Briefly, a 4-methylbenzhydrylamine-functionalized resin (MBHA-HCl, **2**)



Scheme 1. Reagents and conditions: a) 10% DIPEA, CH₂Cl₂, RT, 5 min; b) Boc-L-Pro-OH (**3**), TBTU, DMF, 10% DIPEA in CH₂Cl₂, RT, 2 h; c) 20% TFA in CH₂Cl₂, RT, 20 min; d) Boc-L-His(1-methyl-2-alkyl)-OH (**6a–g**), TBTU, DMF, 10% DIPEA in CH₂Cl₂, RT, 2 h; e) Z-L-pGlu-OH (**8**), TBTU, 10% DIPEA in CH₂Cl₂, RT, 2 h; f) TFMSA, EDT, thioanisole, TFA, 4 °C–RT, 2 h.

was neutralized with *N,N*-diisopropylethylamine (DIPEA) and subsequently coupled with the preformed *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU)-activated ester of Boc-L-Pro-OH (**3**) in *N,N*-dimethylformamide (DMF) to produce a protected amino acid linked resin. The resin-bound Boc-protected amino acid was deprotected with trifluoroacetic acid (TFA), followed by neutralization with DIPEA, to provide free amino acid linked resin **5**. This was then subjected to further coupling and deprotection cycles with Boc-L-His(1-methyl-2-alkyl)-OH **6a–g** and Z-L-pGlu-OH **8**, respectively, to yield peptide resin **9**. The intermediate coupling steps were monitored by Kaiser's test. Cleavage of the peptide from the resin support was carried out by using a solution of trifluoromethanesulfonic acid (TFMSA) in TFA in the presence of thioanisole and 1,2-ethanedithiol (EDT) as scavengers to yield peptide amides **10a–g**.

All subsequent peptides were synthesized by solution-phase synthesis to decrease consumption of the large excess of modified amino acids required for solid-phase synthesis. Reaction of L-Pro-OH **11** with Boc-L-His(1,2-dialkyl)-OH compounds **6a–m** in the presence of 1,3-diisopropylcarbodiimide (DIC) and *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide (HONB) in DMF yielded dipeptides **12a–m** (Scheme 2). Deprotection of the Boc group with TFA produced dipeptide salts **13a–m**. The free dipeptides were obtained *in situ* by reaction of **13a–m**



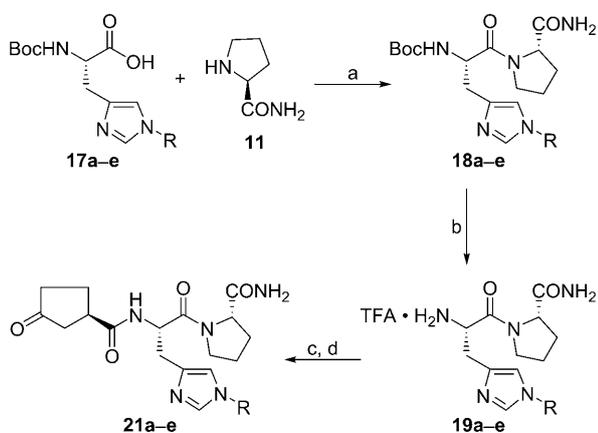
Scheme 2. Reagents and conditions: a) DIC, HONB, DMF, 4 °C, 36 h; b) 40% TFA, CH₂Cl₂, 0 °C, 30 min; c) 7 N NH₃ in CH₃OH, 0 °C, 10 min; d) L-pGlu-OTcp (**14**) or L-pAad-OPfp (**15**), DMF, 4 °C, 36 h.

with a solution of ammonia. The intermediate dipeptides were not isolated; their immediate coupling with L-pGlu-OTcp (**14**) or L-pAad-OPfp (**15**) in DMF produced the desired TRH analogues (**10h-n** and **16a-m**, respectively series **1** and **2**).

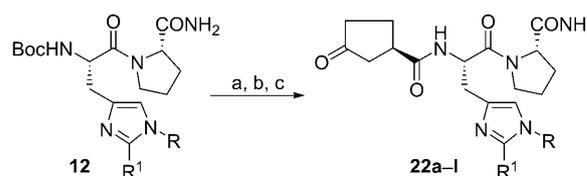
We encountered low yields in using a similar strategy to carry out the synthesis of TRH analogues **21a-e** and **22a-l** (series **3** and **4**). In these cases, coupling of dipeptides **12** or **19** with (1*R*)-3-Ocp-OH (**20**) using DIC and HONB provided a complex mixture containing tripeptides in poor yields. We observed that the use of 1-hydroxybenzotriazole (HOBt) as an auxiliary nucleophile in place of HONB provided better results. Thus, reaction of dipeptides **12** or **19** with **20** using DIC/HOBt produced tripeptides **21a-e** and **22a-l** in satisfactory yields (Scheme 3 and Scheme 4).

Pharmacology at TRH-R1 and TRH-R2

Synthesized TRH analogues were examined for their affinities for TRH-R1 and TRH-R2 and their abilities to serve as agonists



Scheme 3. Reagents and conditions: a) DIC, HONB, DMF, 4 °C, 36 h; b) 40% TFA, CH₂Cl₂, 0 °C, 30 min; c) 7 N NH₃ in CH₃OH, 0 °C, 10 min; d) (1*R*)-3-Ocp-OH (**20**), DIC, HOBt, DMF, 4 °C, 36 h.



Scheme 4. Reagents and conditions: a) 40% TFA, CH₂Cl₂, 0 °C, 30 min; b) 7 N NH₃ in CH₃OH, 0 °C, 10 min; c) (1*R*)-3-Ocp-OH (**20**), DIC, HOBt, DMF, 4 °C, 36 h.

of the receptors.^[36] Affinities, reported as K_i values (μM), were determined by measuring the concentration of the analogue required to compete with [³H][N τ (1)-Me-His]TRH at 2 nM for receptor binding. [N τ (1)-Me-His]TRH is known to bind TRH-R1 and TRH-R2 with affinities higher than TRH. The agonist behavior of the analogues was tested in HEK293EM cells stably expressing TRH-R1 or TRH-R2 by incubating the cells with various doses of the analogues as described.^[21,37] The extent of agonist behavior was then determined by measuring signaling through a reporter gene, and the data are reported as EC₅₀ values (μM) in Table 1.

TRH analogues of series **1** generally exhibited weak binding affinities for TRH-R1 and TRH-R2 (Table 1). Analogue **10a** (R = C₃H₇) displayed modest affinities for TRH-R1 and TRH-R2 (K_i = 0.32 and 0.17 μM , respectively), resulting in a ~16–17-fold decrease in binding affinity relative to TRH. However, **10a** displayed promising agonist potency at TRH-R2 (EC₅₀ = 0.027 μM) over TRH-R1 (EC₅₀ = 0.29 μM) and has 11-fold selectivity for TRH-R2. Introduction of a bulkier and more lipophilic substituent at the C2 position of the His residue (compounds **10b-e**) yielded ligands with poor binding affinities and selectivities. Interestingly, analogue **10f**, with a cyclohexyl group at the C2 position of His, exhibited moderate binding affinity (TRH-R1: K_i = 0.34 μM ; TRH-R2: K_i = 0.14 μM) similar to that of **10a**, but is not a selective agonist (TRH-R1: EC₅₀ = 0.34 μM ; TRH-R2: EC₅₀ = 0.48 μM). To probe the steric requirements, we replaced the 1-methyl group in analogues **10a-f** with a 1-benzyl group. The resulting TRH analogues **10h-n** were found to be inactive at both receptor subtypes. Replacement of pGlu with pAad and attachment of a 1,2-dialkyl group on the His residue also resulted in inactive analogues, **16a-m**. These results provide an initial insight into the minimum and maximum size requirement of the groups placed on the His residue in combination with pGlu and its replacement by pAad in TRH peptides.

Simultaneous replacement of the His residue with 1-alkyl- and 1,2-dialkyl-His residues, and pGlu with its bioisosteric replacement (1*R*)-3-Ocp, provided ligands with the best results (series **3** and **4**). Placement of a methyl group at the N τ (1) position of His provided analogue **21a** [R = (1*R*)-3-Ocp, R¹ = CH₃, R² = H], which exhibits high binding affinities (K_i = 0.17 and K_i = 0.016 μM at TRH-R1 and TRH-R2, respectively). Moreover, analogue **21a** displays potent agonist activity at TRH-R2 with high potency (EC₅₀ = 0.0021 μM), which is ~1.4-fold higher than that of TRH, and exhibits 24-fold selectivity for TRH-R2. Replacement of a methyl group with an ethyl group as in analogue **21b** [R = (1*R*)-3-Ocp, R¹ = CH₃, R² = C₂H₅] resulted in higher

Table 1. Binding affinity (K_i) and signaling (activation) potencies (EC_{50}) produced by TRH analogues (series 1–4) for TRH-R1 and TRH-R2.

Compd	R	R ¹	R ²	K_i [μ M] ^[a]		Fold Selectivity	EC_{50} [μ M] ^[b]		Fold Selectivity	
				TRH-R1	TRH-R2		TRH-R1	TRH-R2		
Series 1:										
10a	L-pGlu	CH ₃	C ₃ H ₇	0.32 (0.24–0.35)	0.17 (0.11–0.23)	1.9	0.29 (0.18–0.47)	0.027 (0.015–0.050)	11	
10b	L-pGlu	CH ₃	CH(CH ₃) ₂	> 50	> 50	–	> 50	> 50	–	
10c	L-pGlu	CH ₃	C(CH ₃) ₃	> 50	> 50	–	> 50	> 50	–	
10d	L-pGlu	CH ₃	c-C ₃ H ₅	11.0 (9.00–13.2)	> 50	4.5	> 50	> 50	–	
10e	L-pGlu	CH ₃	c-C ₅ H ₉	> 50	> 50	–	> 50	> 50	–	
10f	L-pGlu	CH ₃	c-C ₆ H ₁₁	0.34 (0.28–0.38)	0.14 (0.10–0.22)	2.4	0.34 (0.18–0.51)	0.48 (0.31–0.68)	1.4	
10g	L-pGlu	CH ₃	adamantan-1-yl	> 50	> 50	–	> 50	> 50	–	
10h	L-pGlu	CH ₂ C ₆ H ₅	C ₃ H ₇	–	–	–	–	–	–	
10i	L-pGlu	CH ₂ C ₆ H ₅	CH(CH ₃) ₂	> 50	> 50	–	> 50	> 50	–	
10j	L-pGlu	CH ₂ C ₆ H ₅	C(CH ₃) ₃	> 50	> 50	–	> 50	> 50	–	
10k	L-pGlu	CH ₂ C ₆ H ₅	c-C ₃ H ₅	–	–	–	–	–	–	
10l	L-pGlu	CH ₂ C ₆ H ₅	c-C ₅ H ₉	–	–	–	–	–	–	
10m	L-pGlu	CH ₂ C ₆ H ₅	c-C ₆ H ₁₁	> 50	> 50	–	> 50	> 50	–	
10n	L-pGlu	CH ₂ C ₆ H ₅	adamantan-1-yl	> 50	> 50	–	> 50	> 50	–	
Series 2:										
16a	L-pAad	CH ₂ C ₆ H ₅	C ₃ H ₇	> 50	> 50	–	> 50	> 50	–	
16b	L-pAad	CH ₂ C ₆ H ₅	CH(CH ₃) ₂	–	–	–	–	–	–	
16c	L-pAad	CH ₂ C ₆ H ₅	C(CH ₃) ₃	–	–	–	–	–	–	
16d	L-pAad	CH ₂ C ₆ H ₅	c-C ₃ H ₅	> 50	> 50	–	> 50	> 50	–	
16e	L-pAad	CH ₂ C ₆ H ₅	c-C ₅ H ₉	–	–	–	–	–	–	
16f	L-pAad	CH ₂ C ₆ H ₅	c-C ₆ H ₁₁	> 50	> 50	–	> 50	> 50	–	
16g	L-pAad	CH ₂ C ₆ H ₅	adamantan-1-yl	> 50	> 50	–	> 50	> 50	–	
16h	L-pAad	CH ₃	C ₃ H ₇	–	–	–	–	–	–	
16i	L-pAad	CH ₃	CH(CH ₃) ₂	–	–	–	–	–	–	
16j	L-pAad	CH ₃	C(CH ₃) ₃	> 50	> 50	–	> 50	> 50	–	
16k	L-pAad	CH ₃	c-C ₃ H ₅	–	–	–	–	–	–	
16l	L-pAad	CH ₃	c-C ₅ H ₉	> 50	> 50	–	> 50	> 50	–	
16m	L-pAad	CH ₃	adamantan-1-yl	–	–	–	–	–	–	
Series 3:										
21a	(R)-Ocp	CH ₃	H	0.17 (0.11–0.26)	0.016 (0.007–0.027)	10.6	0.05 (0.03–0.09)	0.0021 (0.0009–0.0035)	24	
21b	(R)-Ocp	C ₂ H ₅	H	0.10 (0.07–0.14)	0.32 (0.28–0.37)	3.2	0.14 (0.06–0.20)	0.030 (0.012–0.051)	4.7	
21c	(R)-Ocp	C ₃ H ₇	H	8.1 (6.1–13.2)	12.4 (6.8–16.4)	1.5	4.6 (3.7–9.1)	1.2 (0.54–2.22)	4	
21d	(R)-Ocp	CH(CH ₃) ₂	H	> 50	> 50	–	> 50	> 50	–	
21e	(R)-Ocp	CH ₂ C ₆ H ₅	H	10.2 (8.1–12.2)	14.4 (10.8–20.6)	1.4	3.5 (2.5–7.8)	1.4 (0.44–2.2)	2.5	
Series 4:										
22a	(R)-Ocp	CH ₂ C ₆ H ₅	C ₃ H ₇	0.08 (0.03–0.14)	0.35 (0.27–0.45)	4.4	0.10 (0.04–0.18)	0.015 (0.011–0.047)	6.6	
22b	(R)-Ocp	CH ₂ C ₆ H ₅	CH(CH ₃) ₂	9.6 (5.1–12.2)	10.2 (6.4–15.4)	1	3.6 (3.2–8.1)	1.1 (0.32–1.30)	3.2	
22c	(R)-Ocp	CH ₂ C ₆ H ₅	C(CH ₃) ₃	–	–	–	–	–	–	
22d	(R)-Ocp	CH ₂ C ₆ H ₅	c-C ₃ H ₅	–	–	–	–	–	–	
22e	(R)-Ocp	CH ₂ C ₆ H ₅	c-C ₅ H ₉	–	–	–	–	–	–	
22f	(R)-Ocp	CH ₂ C ₆ H ₅	c-C ₆ H ₁₁	7.8 (6.4–12.7)	9.3 (6.5–12.8)	1.2	2.7 (1.5–6.2)	0.97 (0.52–1.34)	2.7	
22g	(R)-Ocp	CH ₂ C ₆ H ₅	adamantan-1-yl	–	–	–	–	–	–	
22h	(R)-Ocp	CH ₃	C ₃ H ₇	> 50	> 50	–	> 50	> 50	–	
22i	(R)-Ocp	CH ₃	CH(CH ₃) ₂	> 50	> 50	–	–	> 50	–	
22j	(R)-Ocp	CH ₃	C(CH ₃) ₃	–	–	–	–	–	–	
22k	(R)-Ocp	CH ₃	c-C ₃ H ₅	–	–	–	–	–	–	
22l	(R)-Ocp	CH ₃	c-C ₅ H ₉	–	–	–	–	–	–	
22l	(R)-Ocp	CH ₃	adamantan-1-yl	> 50	> 50	–	> 50	> 50	–	
TRH				0.02 (0.01–0.03)	0.02 (0.01–0.03)	–	0.01 (0.008–0.015)	0.003 (0.002–0.007)	–	
[N τ (1)-Me-His]-TRH				0.003 (0.002–0.005)	0.003 (0.002–0.005)	–	0.0021 (0.002–0.005)	0.0002 (0.0002–0.0005)	–	

[a] For binding, cells were incubated with 1 nM [³H]N τ (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 transfected with 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 HEK293 cells. All data represent the mean \pm SD of duplicate determinations in three experiments; –: not determined.

binding affinity for TRH-R1 ($K_i=0.10\ \mu\text{M}$). Analogue **21b** exhibited agonist potency ($\text{EC}_{50}=0.030\ \mu\text{M}$) lower than that of **21a** and was moderately selective for TRH-R2 with 4.7-fold selectivity. The binding affinities and activation potencies were further decreased with bulkier substituents placed at the N τ (1) position of the His residue (analogues **21c** and **21e**). These analogues also exhibited modest selectivity of 2.5–4-fold for TRH-R2. These results are consistent with our previous findings that the ring NH group of pGlu is not necessary for the binding of the peptide to TRH receptors, and that modulation of the His residue at the N1 or C2 positions imparts selectivity toward TRH-R2.^[20–24]

Finally, TRH analogues **22a–g** (series 4) in which the His residue is replaced with 1-benzyl-2-alkyl-His and pGlu is replaced with (1*R*)-3-Ocp exhibited moderate binding affinities and activation potencies. For example, analogue **22a** [$R=(1R)\text{-3-Ocp}$, $R^1=\text{CH}_2\text{C}_6\text{H}_5$, $R^2=\text{CH}_2\text{CH}_2\text{CH}_3$] exhibited K_i values of 0.08 and $0.35\ \mu\text{M}$ to TRH-R1 and TRH-R2, respectively. Similarly, analogues **22b** [$R=(1R)\text{-3-Ocp}$, $R^1=\text{C}_6\text{H}_5\text{CH}_2$, $R^2=\text{CH}(\text{CH}_3)_2$] and **22f** [$R=(1R)\text{-3-Ocp}$, $R^1=\text{C}_6\text{H}_5\text{CH}_2$, $R^2=c\text{-C}_6\text{H}_{11}$] exhibited modest binding affinities for TRH-R1 and TRH-R2. Analogue **22a** also exhibited modest activation potency ($\text{EC}_{50}=0.015\ \mu\text{M}$) and has 6.6-fold specificity for TRH-R2. Analogues **22b** and **22f** were also found to have 3.2- and 2.7-fold respective selectivity in activation potencies (EC_{50}) for TRH-R2. All TRH analogues **22h–l**, in which His is replaced with 1-methyl-2-alkyl-His, and pGlu is replaced with (1*R*)-3-Ocp, were found to be inactive at both TRH receptors (Table 1).

Antagonism of pentobarbital-induced sleeping time

One of the best documented CNS effects of TRH is its analeptic action manifested by the decrease in barbiturate narcosis. TRH is an effective analeptic agent, which reduces pentobarbital-induced sleeping time by 50% or more following peripheral administration of high doses or central injection of lower doses in rats, rabbits, and monkeys.^[38,39] The synthesized TRH analogues were evaluated in vivo by using the antagonism of a pentobarbital-induced sleeping time model as described.^[40] TRH analogues were injected intravenously through the tail vein at a dose of $10\ \mu\text{mol kg}^{-1}$ (equivalent to $3.7\ \text{mg kg}^{-1}$ TRH). Ten minutes after administration of the TRH analogue, each animal received $50\ \text{mg kg}^{-1}$ sodium pentobarbital intraperitoneally. The sleeping time was recorded as the time elapsed from the onset of loss of righting reflex until it returned (Table 2).

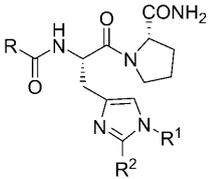
Analogues **10a**, **10b**, **10d**, **10e**, and **10f** (series 1) showed a significant decrease in sleeping time as compared with the control group; however, their effects were not significantly different from those of the TRH-treated group. All other analogues of this series were ineffective in decreasing sleep time. Similarly, none of the analogues from series 2 showed positive results in decreasing sleep time. Interestingly, some of the analogues from series 1 and 2 were found to increase the sleeping time significantly over control and TRH. In particular, analogues **10h**, **10i**, **10j**, **10k**, **10l**, **10m**, and **10n** of series 1, and **16d** of series 2 increased the sleeping time significantly relative to the

control group. The observed potentiation of pentobarbital-induced sleep activity may be caused by alterations in receptor interactions due to the specific properties of the substitution on the His residue of the TRH analogues. This altered interaction may potentiate the action of pentobarbital, possibly by γ -aminobutyric acid (GABA)-mediated postsynaptic inhibition through an allosteric modification of GABA receptors, or by enhancement of K^+ conductance or inhibition of Ca^{2+} conductance in neurons.^[41–48] Most of the synthesized analogues of series 3 and 4 showed positive results when evaluated in vivo for antagonism of pentobarbital-induced sleeping time in mice. The sleeping time after administration of analogues **21a**, **21b**, **21c**, **22a**, **22b**, **22f**, and **22l** were 28.2 ± 1.7 , 27.8 ± 4.8 , 34.4 ± 1.4 , 26.6 ± 1.1 , 32.6 ± 6.1 , 31.7 ± 2.3 , and 32.6 ± 5.5 min, respectively. The decrease in sleeping time caused by these an-

Table 2. Results of pentobarbital-induced sleeping time experiment in mice for TRH analogues (series 1–4).^[a]

Compd	R	R ¹	R ²	Sleep	
				Time [min]	Decrease in Sleep Time [%]
Series 1:					
10a	L-pGlu	CH ₃	C ₃ H ₇	41.7 ± 6.1 ^[b]	52.7
10b	L-pGlu	CH ₃	CH(CH ₃) ₂	66.2 ± 9.8	25.1
10c	L-pGlu	CH ₃	C(CH ₃) ₃	59.6 ± 5.5	32.5
10d	L-pGlu	CH ₃	<i>c</i> -C ₃ H ₅	51.4 ± 5.1 ^[b]	41.8
10e	L-pGlu	CH ₃	<i>c</i> -C ₅ H ₉	48.2 ± 10.1 ^[b]	45.4
10f	L-pGlu	CH ₃	<i>c</i> -C ₆ H ₁₁	43 ± 8.1 ^[b]	51.3
10g	L-pGlu	CH ₃	adamantan-1-yl	66.9 ± 5.3	24.2
10h	L-pGlu	CH ₂ C ₆ H ₅	C ₃ H ₇	105.1 ± 5.76	−18.8
10i	L-pGlu	CH ₂ C ₆ H ₅	CH(CH ₃) ₂	157.2 ± 17 ^[b,c]	−77.8
10j	L-pGlu	CH ₂ C ₆ H ₅	C(CH ₃) ₃	141.9 ± 23.9 ^[b,c]	−60.5
10k	L-pGlu	CH ₂ C ₆ H ₅	<i>c</i> -C ₃ H ₅	147.1 ± 12.7 ^[b,c]	−66.4
10l	L-pGlu	CH ₂ C ₆ H ₅	<i>c</i> -C ₅ H ₉	163.3 ± 18.7 ^[b,c]	−84.7
10m	L-pGlu	CH ₂ C ₆ H ₅	<i>c</i> -C ₆ H ₁₁	136.9 ± 26.7 ^[b,c]	−54.9
10n	L-pGlu	CH ₂ C ₆ H ₅	adamantan-1-yl	223.6 ± 22.3 ^[b,c]	−152.9
Series 2:					
16a	L-pAad	CH ₂ C ₆ H ₅	C ₃ H ₇	69.3 ± 7.3	22.8
16b	L-pAad	CH ₂ C ₆ H ₅	CH(CH ₃) ₂	ND	–
16c	L-pAad	CH ₂ C ₆ H ₅	C(CH ₃) ₃	ND	–
16d	L-pAad	CH ₂ C ₆ H ₅	<i>c</i> -C ₃ H ₅	250.5 ± 23.1	−178.7
16e	L-pAad	CH ₂ C ₆ H ₅	<i>c</i> -C ₅ H ₉	95.5 ± 4.7	−6.2
16f	L-pAad	CH ₂ C ₆ H ₅	<i>c</i> -C ₆ H ₁₁	81.0 ± 3.1	9.8
16g	L-pAad	CH ₂ C ₆ H ₅	adamantan-1-yl	87.5 ± 5.0	2.6
16h	L-pAad	CH ₃	C ₃ H ₇	98.1 ± 9.4	−9.1
16i	L-pAad	CH ₃	CH(CH ₃) ₂	124.3 ± 5.8	−38.2
16j	L-pAad	CH ₃	C(CH ₃) ₃	93.1 ± 4.5	−3.6
16k	L-pAad	CH ₃	<i>c</i> -C ₃ H ₅	108.1 ± 14.4	−20.2
16l	L-pAad	CH ₃	<i>c</i> -C ₆ H ₁₁	103.2 ± 9.1	−14.8
16m	L-pAad	CH ₃	adamantan-1-yl	76.0 ± 10.9	15.4
Series 3:					
21a	(<i>R</i>)-Ocp	CH ₃	H	28.2 ± 1.7 ^[b,c]	68.6
21b	(<i>R</i>)-Ocp	C ₂ H ₅	H	27.8 ± 4.8 ^[b,c]	69
21c	(<i>R</i>)-Ocp	C ₃ H ₇	H	34.5 ± 1.4 ^[b,c]	61.6
21d	(<i>R</i>)-Ocp	CH(CH ₃) ₂	H	123.2 ± 5.9	−36.8
21e	(<i>R</i>)-Ocp	CH ₂ C ₆ H ₅	H	35.8 ± 1.9 ^[b,c]	60.1

Table 2. (Continued)



Compd	R	R ¹	R ²	Sleep Time [min]	Decrease in Sleep Time [%]
Series 4:					
22a	(R)-Ocp	CH ₂ C ₆ H ₅	C ₃ H ₇	26.6 ± 1.1 ^[b,c]	70.3
22b	(R)-Ocp	CH ₂ C ₆ H ₅	CH(CH ₃) ₂	32.7 ± 6.1 ^[b,c]	63.6
22c	(R)-Ocp	CH ₂ C ₆ H ₅	C(CH ₃) ₃	91.6 ± 3.8	-1.9
22d	(R)-Ocp	CH ₂ C ₆ H ₅	c-C ₃ H ₅	104.9 ± 5.5	-16.7
22e	(R)-Ocp	CH ₂ C ₆ H ₅	c-C ₅ H ₉	ND	-
22f	(R)-Ocp	CH ₂ C ₆ H ₅	c-C ₆ H ₁₁	31.7 ± 2.3 ^[b,c]	64.7
22g	(R)-Ocp	CH ₂ C ₆ H ₅	adamantan-1-yl	ND	-
22h	(R)-Ocp	CH ₃	C ₃ H ₇	80.2 ± 2.4	10.7
22i	(R)-Ocp	CH ₃	CH(CH ₃) ₂	39.8 ± 5.5 ^[b,c]	55.6
22j	(R)-Ocp	CH ₃	C(CH ₃) ₃	89.8 ± 3.0	-0.01
22k	(R)-Ocp	CH ₃	c-C ₆ H ₁₁	106.2 ± 3.0	-18.1
22l	(R)-Ocp	CH ₃	adamantan-1-yl	32.6 ± 5.5 ^[b,c]	63.6
control				89.8 ± 6.8	0.00
TRH				52.6 ± 4 ^[b]	41.4

[a] Pentobarbital was injected (50 mg kg⁻¹ i.p.) 10 min after i.v. injection of the TRH/TRH analogues. Sleep time was recorded as the time elapsed from the onset of loss to regaining of the righting reflex. Six to eight Swiss albino mice (body weight 20–30 g) were used in each group. Values are expressed as the mean ± SEM; ND: not determined. [b] $p < 0.05$ relative to control using one-way ANOVA followed by post hoc Tukey test. [c] $p < 0.05$ relative to TRH using one-way ANOVA followed by post hoc Tukey test.

alogues was significantly different from control (89.1 ± 4.6 min) as well as from the TRH-treated group (52.6 ± 3.5 min). Analogues **21a** and **21b** of series **3** and **22a** of series **4** were found to be the most potent analogues, effecting decreases in sleep time of 68.3, 68.8, and 70.1% relative to control. The analeptic effects of these TRH analogues may be due to the enhancement of arousal or excitatory activity of TRH. TRH is well known for this activity, which may be mediated through neuromodulation of various neurotransmitters such as acetylcholine, as it directly affects the septohippocampal cholinergic pathway.^[1,2] In addition to cholinergic activity, blockade of leaking K⁺ current has also been proposed for this particular activity.^[49]

As evident from the receptor binding studies and preliminary in vivo screening using the pentobarbital-based sleep time assay, TRH peptides that show good analeptic activity bind with moderate affinities to TRH-R1 and TRH-R2, but exhibit selective activation at TRH-R2, supporting our hypothesis that TRH-R2-selective analogues exhibit potent CNS effects. The outcome of these results show that several TRH analogues (**21a**, **21b**, **21c**, **22a**, **22b**, **22f**, and **22l**) are more potent than TRH in their ability to decrease pentobarbital-induced sleeping time in mice, which in turn prompted us to undertake further evaluation of these compounds in various models of epilepsy. The analogues were selected on the basis of their observed biological activity and availability.

Anticonvulsant activity

Previous pharmacological studies have demonstrated that TRH and its analogues inhibit pentylenetetrazole (PTZ)- or glutamate-induced seizures in rat.^[17,50] Therefore, selected compounds **21a–c** and **22a,b** were evaluated for anticonvulsant activity against PTZ-induced seizures in mice according to a method described earlier.^[51] The compounds were dissolved in normal saline and administered at doses of 10 μmol kg⁻¹ i.v. After 10 min, animals were treated with PTZ (65 mg kg⁻¹ i.p.) and observed for 30 min after PTZ challenge. The results of these experiments are listed in Table 3. Compounds **21a,b** and **22a,b** provided significant protection against clonic seizures

Table 3. Effect of TRH and analogues on pentylenetetrazole (PTZ) and maximal electric shock (MES)-induced seizures.^[a]

Compd	PTZ: Clonic Convulsions		MES: Hind Limb Extension Duration [s]
	Onset [s]	Frequency [min ⁻¹]	
saline	67 ± 5	1.3 ± 0.16	13.6 ± 1.10
TRH	79 ± 7	1.3 ± 0.49	11.2 ± 0.50
21a	153 ± 7 ^{*,**}	1.3 ± 0.33	10.0 ± 0.60
21b	149 ± 8 ^{*,**}	1.3 ± 0.21	11.3 ± 1.4
21c	68 ± 3	1.3 ± 0.21	12.3 ± 0.90
22a	179 ± 8 ^{*,**}	1.60 ± 0.06	12.0 ± 0.40
22b	224 ± 53 ^{*,**}	1.20 ± 0.20	11.0 ± 0.70

[a] Mice (6–9 per group) were treated with saline, TRH, or compounds (**21a–c** and **22a,b**) at 10 μmol kg⁻¹ i.v. 10 min prior to administration of PTZ at 65 mg kg⁻¹ i.p. or MES seizure, and were observed for 1 h. Values are expressed as the mean ± SEM; statistical analysis was done with one-way ANOVA followed by Tukey test: * $p < 0.05$ versus saline and ** $p < 0.05$ versus TRH-treated group.

induced by PTZ (between 224 ± 5 and 149 ± 8 s, compared with TRH: 79 ± 7 s), whereas analogue **21c** is inactive and did not show any protection of PTZ-induced seizures. The selected TRH analogues were also evaluated for maximal electric shock (MES)-induced epilepsy using a protocol described earlier.^[52] Treatment with TRH analogues at doses of 10 μmol kg⁻¹ i.v. was done 10 min prior to the administration of MES at 45 mA for 0.2 s. The duration of tonic hind limb extension was recorded after MES, and the results are listed in Table 3. None of the tested analogues showed protection in the MES model. The effectiveness of TRH analogues in PTZ- but not in MES-induced seizure indicates their potential in clinical absence seizures. TRH is known to provide protection against seizures induced by kainic acid and glutamate, supporting the observed potency of TRH analogues in PTZ-induced seizures.^[50,53] TRH was also shown to increase GABA release, and this may contribute to its antiepileptic action.^[54] However, additional studies are required to explore the exact mechanism of action of these analogues.

Conclusions

In the present study, we probed the structural requirements of His and pGlu residues in receptor binding and biological activity by synthesizing new TRH analogues in which His residue

was replaced with 1-alkyl-L-His/1,2-dialkyl-L-His; while pGlu residue was either retained or replaced with (1*R*)-3-Ocp/L-pAad. Analogues in which pGlu residue was replaced with (1*R*)-3-Ocp and His was substituted at the N-1/C-2 positions displayed the best pharmacological profile. Compounds **21 a**, **21 b**, and **22 a** were all found to be full agonists with good to high functional selectivity for TRH-R2. At the same time, analogue **21 a** exhibited highest binding affinity among the tested analogues. Analeptic activity evaluation using pentobarbital-induced sleeping time model revealed that some of the tested analogues were more potent compared than TRH. Analogues **21 a**, **21 b**, **21 c**, **22 a**, **22 b** and **22 f** produced 61 to 70% reduction in sleeping time compared to control. The best analeptic profile was displayed by analogues **21 a**, **21 b**, and **22 a** by reducing the sleeping time to almost 50% more than TRH. To our surprise, some analogues like **10 l**, **10 n** and **16 d** increased the sleeping time significantly. The results of this study indicate TRH-R2 as a plausible receptor subtype mediating the analeptic effects of TRH and its analogues. Analogues **21 a**, **21 b**, **21 c**, **22 a**, and **22 b** were also investigated against PTZ- and MES-induced seizures in mice, which reflect the generalized and partial seizures that commonly occur in humans. Compounds **21 a**, **21 b**, **22 a** and **22 b** provide significant protection against PTZ-induced clonic seizures. However, all analogues failed to show protection against seizures induced by MES, suggesting their beneficial effects in the generalized form of seizures. To what extent the systemically active TRH analogues may have improved the BBB permeability remains unanswered. As clear from the results of receptor binding and in vivo evaluation, TRH peptides (**21 a**, **21 b**, **22 a** and **22 b**) showing good analeptic/anticonvulsant activity bind with moderate affinities to TRH-R1 and TRH-R2, but exhibited selectivities for TRH-R2, which is possibly responsible for the CNS effects of TRH. In conclusion, these analogues are potent and functionally selective ligands and displayed potent analeptic and anticonvulsant activity when tested in vivo in mice. For the first time, we have shown that TRH-R2 selective analogues displayed potent CNS profile (antiepileptic activity), thereby making them promising candidates for further development for the prophylactic management of epilepsy.

Experimental Section

Chemicals

Amino acids used in this study were purchased from Novabiochem (Germany) or ChemImpex International (Wood Dale, IL, USA). All other chemicals were purchased from Aldrich Chemical Ltd. (Milwaukee, WI, USA). Solvents used for peptide synthesis purification were acquired from commercial sources, were of analytical or HPLC grade, and were used without further purification unless otherwise stated.

Materials and methods

Peptides were routinely checked for their purity on pre-coated silica gel G₂₅₄ TLC plates (Merck), and the spots were visualized by UV light and then by exposure to I₂ vapors. Column chromatogra-

phy was carried out on Merck silica gel (230–400 mesh) or neutral alumina. IR spectra ($\bar{\nu}_{\max}$ in cm⁻¹) were recorded on a Nicolet FTIR Impact 410 instrument either as neat or KBr pellets. ¹H and ¹³C NMR spectra were recorded on a 300 MHz Bruker FTNMR (Avance DPX 300) spectrometer using tetramethylsilane (TMS) as internal standard, and chemical shift values (δ) are reported in ppm. MS data were recorded on an HRMS (Finnigan Mat LCQ) spectrometer (APCI/ESI). Elemental analyses were recorded on an Elementar Vario EL spectrometer. The results of elemental analyses were within $\pm 0.4\%$ of theoretical values. Optical rotations were recorded on a PerkinElmer 241MC polarimeter. All final peptides were checked for their homogeneity on a Shimadzu SPD-M20A HPLC system using a SupelcosilTM LC-8 column, 5 μ m (25 cm \times 4.6 mm i.d.). The peptides were analyzed by using an isocratic solvent system of CH₃CN/H₂O/TFA (ratios as indicated) at a flow rate of 1 mL min⁻¹.

General method for the synthesis of L-pGlu-L-His(1-methyl-2-alkyl)-L-ProNH₂ (10 a–g)

4-Methylbenzhydrylamine (MBHA-HCl) resin (**2**, 300 mg, 0.31 mmol) was swelled in CH₂Cl₂ (5 mL) for 5 min, and then neutralized with DIPEA (10% solution in CH₂Cl₂) for 5 min followed by washing with DMF (2 \times 10 mL) and CH₂Cl₂ (10 mL). The reaction of the first amino acid Boc-L-Pro-OH (**3**) was done in the presence of the coupling reagent TBTU and activator DIPEA (10% solution in CH₂Cl₂) for 2 h. The Boc group was cleaved with TFA (20% solution in CH₂Cl₂) for 20 min. The amino acid linked resin was again neutralized with DIPEA as described above to afford **5**. Reaction of **5** with pre-formed TBTU esters of Boc-L-His(1-methyl-2-alkyl)-OH **6 a–g** for 2 h in DMF followed by removal of Boc group with TFA (20% solution in CH₂Cl₂) and finally neutralization with DIPEA (10% in CH₂Cl₂) afforded dipeptides **7 a–g**. Upon coupling with Z-L-pGlu-OH (**8**) as described above, the dipeptides **7 a–g** produced desired peptide resins **9 a–g**. All coupling reactions were monitored quantitatively by Kaiser's test for completion. The dry peptide-linked resin **9 a–g** was taken in a two-necked round-bottom flask equipped with a drying tube and a rubber septum. A cleavage cocktail of EDT (1 mL), thioanisole (2 mL), TFA (10 mL), and TFMSA (1.0 mL) were added to the reaction vessel through the septum, and reaction mixture was stirred for 10 min at 4 °C followed by stirring at ambient temperature for another 2 h. The crude peptide was separated from the solid support by filtration, and the resin was washed with TFA (3 \times 4 mL). The solvent was removed under reduced pressure, and the residue was neutralized with a saturated solution of ammonium bicarbonate. The nonpolar impurities were removed by extracting the aqueous layer with Et₂O (3 \times 10 mL). The aqueous layer was evaporated under reduced pressure to afford crude peptide, which upon purification using column chromatography over neutral alumina using CHCl₃/CH₃OH (4:1) as eluent, produced **10 a–g**.

L-pGlu-L-His(1-methyl-2-propyl)-L-ProNH₂ (**10 a**). mp: 77–80 °C (dec.); ¹H NMR (CD₃OD): δ = 7.16 (s, 1H), 4.90 (m, 1H), 4.46 (m, 1H), 4.21 (m, 1H), 3.79 (m, 1H), 3.73 (s, 3H), 3.54 (m, 1H), 2.95 (m, 2H), 2.87 (t, 2H, *J* = 7.8 Hz), 2.43–1.92 (m, 8H), 1.75 (m, 2H), 1.01 (t, 3H, *J* = 7.8 Hz); ¹³C NMR (CD₃OD): δ = 14.51, 21.72, 24.33, 25.16, 29.33, 29.38, 30.60, 31.83, 32.85, 46.81, 51.13, 55.27, 60.20, 118.47, 135.22, 147.45, 169.46, 171.39, 177.40, 177.59; MS (APCI): *m/z* 419 [M+1]⁺; Anal. calcd for C₂₀H₃₀N₆O₄ (418.5): C 57.40, H 7.23, N 20.08, found: C 57.82, H 6.77, N 20.37; *R*_f = 0.57 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: *t*_R = 4.60 min, purity: 92.2% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pGlu-L-His(1-methyl-2-isopropyl)-L-ProNH₂ (10b). mp: 92–94 °C (dec.); ¹H NMR (CD₃OD): δ = 7.22 (s, 1H), 4.97 (m, 1H), 4.46 (m, 1H), 4.23 (m, 1H), 3.75 (m, 1H), 3.79 (s, 3H), 3.59 (m, 1H), 3.38 (m, 1H), 3.13 (m, 2H), 2.43–1.91 (m, 8H), 1.40 (m, 6H); ¹³C NMR (CD₃OD): δ = 22.36, 24.33, 25.16, 29.18, 29.23, 33.11, 46.81, 51.20, 55.27, 60.20, 118.16, 136.32, 152.94, 169.66, 171.20, 177.50, 177.70; MS (APCI): *m/z* 419 [M+1]⁺; Anal. calcd for C₂₀H₃₀N₆O₄ (418.5): C 57.40, H 7.23, N 20.08, found: 57.53, H 7.42, N 20.44; *R*_f = 0.56 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: *t*_R = 4.47 min, purity: 95.2% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pGlu-L-His(1-methyl-2-tert-butyl)-L-ProNH₂ (10c). mp: 85–87 °C (dec.); ¹H NMR (CD₃OD): δ = 6.79 (s, 1H), 4.87 (m, 1H), 4.39 (m, 1H), 4.18 (m, 1H), 3.77 (m, 1H), 3.74 (s, 3H), 3.56 (m, 1H), 2.93 (m, 2H), 2.45–1.96 (m, 8H), 1.40 (m, 9H); ¹³C NMR (CD₃OD): δ = 24.40, 25.16, 28.35, 29.23, 29.38, 31.68, 32.56, 35.14, 46.81, 51.28, 55.27, 60.20, 117.97, 135.44, 154.42, 169.67, 170.65, 176.80, 177.21; MS (APCI): *m/z* 433 [M+1]⁺; Anal. calcd for C₂₁H₃₂N₆O₄ (432.2): C 58.32, H 7.46, N 19.43, found: C 58.77, H 7.29, N 19.76; *R*_f = 0.60 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: *t*_R = 4.64 min, purity: 93.8% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pGlu-L-His(1-methyl-2-cyclopropyl)-L-ProNH₂ (10d). mp: 64–66 °C (dec.); ¹H NMR (CD₃OD): δ = 6.81 (s, 1H), 4.76 (m, 1H), 4.38 (m, 1H), 4.17 (m, 1H), 3.77 (m, 1H), 3.65 (s, 3H), 3.49 (m, 1H), 2.88 (m, 2H), 2.44–1.87 (m, 8H), 2.14 (m, 1H), 0.92 (m, 4H); ¹³C NMR (CD₃OD): δ = 7.81, 8.51, 24.33, 25.16, 29.33, 29.38, 31.83, 33.27, 46.81, 51.19, 55.27, 60.20, 117.33, 136.66, 151.70, 169.66, 171.39, 177.40, 177.59; MS (APCI): *m/z* 417 [M+1]⁺; Anal. calcd for C₂₀H₂₈N₆O₄ (416.5): C 57.68, H 6.78, N 20.18, found: C 57.79, H 6.93, N 20.29; *R*_f = 0.62 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: *t*_R = 4.23 min, purity: 97.5% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pGlu-L-His(1-methyl-2-cyclopentyl)-L-ProNH₂ (10e). mp: 100–101 °C (dec.); ¹H NMR (CD₃OD): δ = 6.82 (s, 1H), 4.80 (m, 1H), 4.38 (m, 1H), 4.18 (m, 1H), 3.78 (m, 1H), 3.58 (s, 3H), 3.32 (m, 1H), 3.16 (m, 1H), 2.93 (m, 2H), 2.47–1.26 (m, 16H); ¹³C NMR (CD₃OD): δ = 24.33, 24.76, 25.16, 29.23, 29.38, 31.68, 33.04, 33.42, 46.81, 51.25, 55.27, 60.20, 118.08, 134.20, 150.20, 168.50, 170.65, 176.90, 178.20; MS (APCI): *m/z* 445 [M+1]⁺; Anal. calcd for C₂₂H₃₂N₆O₄ (444.5): C 59.44, H 7.26, N 18.91, found: C 59.81, H 7.62, N 18.73; *R*_f = 0.64 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: *t*_R = 5.80 min, purity: 99.1% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pGlu-L-His(1-methyl-2-cyclohexyl)-L-ProNH₂ (10f). mp: 110–112 °C (dec.); ¹H NMR (CD₃OD): δ = 6.81 (s, 1H), 4.82 (m, 1H), 4.39 (m, 1H), 4.17 (m, 1H), 3.79 (m, 1H), 3.59 (s, 3H), 3.49 (m, 1H), 2.93 (m, 2H), 2.77 (m, 1H), 2.41–1.26 (m, 18H); ¹³C NMR (CD₃OD): δ = 23.98, 25.10, 26.15, 26.85, 29.21, 32.05, 33.09, 34.07, 46.46, 51.10, 54.80, 60.60, 118.00, 134.33, 149.49, 169.66, 171.39, 177.40, 178.10; MS (APCI): *m/z* 459 [M+1]⁺; Anal. calcd for C₂₃H₃₄N₆O₄ (458.6): C 60.24, H 7.47, N 18.33, found: C 60.29, H 7.78, N 17.98; *R*_f = 0.68 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: *t*_R = 7.10 min, purity: 97.1% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pGlu-L-His[1-methyl-2-(adamantan-1-yl)]-L-ProNH₂ (10g). mp: 121–123 °C (dec.); ¹H NMR (CD₃OD): δ = 6.72 (s, 1H), 4.64 (m, 1H), 4.39 (m, 1H), 4.17 (m, 1H), 3.82 (m, 1H), 3.77 (s, 3H), 3.53 (m, 1H), 2.91 (m, 2H), 2.46–2.24 (m, 4H), 2.21–1.81 (m, 19H); ¹³C NMR (CD₃OD): δ = 25.10, 25.1, 29.3, 29.6, 31.80, 35.06, 36.01, 39.55, 40.39, 46.81, 51.34, 55.30, 60.10, 117.94, 135.84, 149.60, 169.67, 171.45, 177.28, 177.60; MS (APCI): *m/z* 511 [M+1]⁺; Anal. calcd for C₂₇H₃₈N₆O₄ (510.6): C 63.51, H 7.50, N 16.46, found: C 63.89, H 7.22,

N 16.91; *R*_f = 0.72 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: *t*_R = 21.22 min, purity: 94.2% [CH₃CN/H₂O/TFA (20:80:0.1%)].

General method for the synthesis of L-pGlu-L-His(1-benzyl-2-alkyl)-L-ProNH₂ (10h–n)

The dipeptide salt (**13a–g**, 1 mmol) was neutralized by addition of a solution of 7 N NH₃ in CH₃OH (10 mL) for 10 min at 0 °C. The solvent was evaporated under reduced pressure to afford free dipeptides. A solution of free peptides (0.75 mmol) in DMF (4 mL) was cooled to 4 °C, and L-pGlu-OTcp (**14**, 0.81 mmol) was added, and the reaction mixture was stirred at 4 °C for 36 h. The solvent was removed under reduced pressure, and the resulting residue was purified by column chromatography over neutral alumina using CHCl₃/CH₃OH (4:1) as eluent to provide tripeptides (**10h–n**).

L-pGlu-L-His(1-benzyl-2-propyl)-L-ProNH₂ (10h). Yield: 18%; mp: 102–104 °C (dec.); ¹H NMR (CD₃OD): δ = 7.33 (m, 3H), 7.18 (m, 2H), 6.95 (s, 1H), 5.14 (s, 2H), 4.80 (m, 1H), 4.41 (m, 1H), 4.18 (m, 1H), 3.78 (m, 1H), 3.42 (m, 1H), 2.98 (m, 2H), 2.62 (t, 2H, *J* = 7.5 Hz), 2.42–1.94 (m, 8H), 1.59 (m, 2H), 0.91 (t, 3H, *J* = 7.2 Hz); MS (APCI): *m/z* 495 [M+1]⁺; Anal. calcd for C₂₆H₃₄N₆O₄ (494.6): C 63.14, H 6.93, N 16.99, found: C 62.95, H 7.08, N 17.14; *R*_f = 0.47 [CH₃OH/25% NH₄OH/CHCl₃ (18:2:80)]; HPLC: *t*_R = 4.05 min, purity: 94.2% [CH₃CN/H₂O/TFA (50:50:0.1%)].

L-pGlu-L-His(1-benzyl-2-isopropyl)-L-ProNH₂ (10i). Yield: 19%; mp: 95–97 °C (dec.); ¹H NMR (CDCl₃): δ = 7.36 (m, 3H), 7.04 (m, 2H), 6.68 (s, 1H), 5.02 (s, 2H), 4.81 (m, 1H), 4.47 (m, 1H), 4.15 (m, 1H), 3.70 (m, 1H), 3.27 (m, 1H), 2.99 (m, 3H), 2.45–1.88 (m, 8H), 1.18 (m, 6H); MS (APCI): *m/z* 495 [M+1]⁺; Anal. calcd for C₂₆H₃₄N₆O₄ (494.6): C 63.14, H 6.93, N 16.99, found: C 63.38, H 7.24, N 16.82; *R*_f = 0.45 [CH₃OH/25% NH₄OH/CHCl₃ (18:2:80)]; HPLC: *t*_R = 4.04 min, purity: 96.8% [CH₃CN/H₂O/TFA (50:50:0.1%)].

L-pGlu-L-His(1-benzyl-2-tert-butyl)-L-ProNH₂ (10j). Yield: 12%; mp: 104–106 °C (dec.); ¹H NMR (CD₃OD): δ = 7.30 (m, 3H), 7.07 (m, 2H), 6.79 (s, 1H), 5.35 (m, 2H), 4.81 (m, 1H), 4.39 (m, 1H), 4.15 (m, 1H), 3.78 (m, 1H), 3.56 (m, 1H), 2.94 (m, 2H), 2.40–1.95 (m, 8H), 1.36 (s, 9H); MS (APCI): *m/z* 509 [M+1]⁺; Anal. calcd for C₂₇H₃₆N₆O₄ (508.3): C 63.76, H 7.13, N 16.52, found: C 64.12, H 6.89, N 16.59; *R*_f = 0.45 [CH₃OH/25% NH₄OH/CHCl₃ (14:2:84)]; HPLC: *t*_R = 4.07 min, purity: 95.1% [CH₃CN/H₂O/TFA (50:50:0.1%)].

L-pGlu-L-His(1-benzyl-2-cyclopropyl)-L-ProNH₂ (10k). Yield: 13%; mp: 84–86 °C (dec.); ¹H NMR (CD₃OD): δ = 7.29 (m, 5H), 6.96 (s, 1H), 5.24 (m, 2H), 4.78 (m, 1H), 4.39 (m, 1H), 4.16 (m, 1H), 3.76 (m, 1H), 3.43 (m, 1H), 2.93 (m, 2H), 2.40–1.93 (m, 8H), 1.83 (m, 1H), 0.90 (m, 4H); MS (APCI): *m/z* 493 [M+1]⁺; Anal. calcd for C₂₆H₃₂N₆O₄ (492.6): C 63.40, H 6.55, N 17.06, found: C 63.44, H 6.69, N 16.94; *R*_f = 0.55 [CH₃OH/25% NH₄OH/CHCl₃ (14:2:84)]; HPLC: *t*_R = 4.02 min, purity: 95.0% [CH₃CN/H₂O/TFA (50:50:0.1%)].

L-pGlu-L-His(1-benzyl-2-cyclopentyl)-L-ProNH₂ (10l). Yield: 27%; mp: 110–112 °C (dec.); ¹H NMR (CD₃OD): δ = 7.30 (m, 3H), 7.15 (m, 2H), 6.92 (s, 1H), 5.15 (s, 2H), 4.80 (m, 1H), 4.39 (m, 1H), 4.16 (m, 1H), 3.77 (m, 1H), 3.47 (m, 1H), 2.96 (m, 3H), 2.30–1.28 (m, 16); MS (APCI): *m/z* 521 [M+1]⁺; Anal. calcd for C₂₈H₃₆N₆O₄ (520.6): C 64.60, H 6.97, N 16.14, found: C 64.89, H 7.11, N 15.98; *R*_f = 0.40 [CH₃OH/25% NH₄OH/CHCl₃ (14:2:84)]; HPLC: *t*_R = 4.58 min, purity: 98.5% [CH₃CN/H₂O/TFA (50:50:0.1%)].

L-pGlu-L-His(1-benzyl-2-cyclohexyl)-L-ProNH₂ (10m). Yield: 23%; mp: 120–122 °C (dec.); ¹H NMR (CDCl₃): δ 7.34 (m, 3H), 7.06 (m,

2H), 6.67 (s, 1H), 5.15 (s, 2H), 4.89 (m, 1H), 4.46 (m, 1H), 4.14 (m, 1H), 3.69 (m, 1H), 3.26 (m, 1H), 2.97 (m, 2H), 2.56 (m, 1H), 2.47–1.22 (m, 18H); MS (APCI): m/z 535 $[M+1]^+$; Anal. calcd for $C_{29}H_{38}N_6O_4$ (534.7): C 65.15, H 7.16, N 15.72, found: C 64.65, H 6.98, N 16.20; $R_f=0.40$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (14:2:84)]; HPLC: $t_R=4.77$ min, purity: 92.8% [$CH_3CN/H_2O/TFA$ (50:50:0.1%)].

L-pGlu-L-His[1-benzyl-2-(adamantan-1-yl)]-L-ProNH₂ (10n). Yield: 13%; mp: 116–118 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.29$ (m, 3H), 7.04 (m, 2H), 6.77 (s, 1H), 5.41 (m, 2H), 4.80 (m, 1H), 4.39 (m, 1H), 4.15 (m, 1H), 3.78 (m, 1H), 3.57 (m, 1H), 2.95 (m, 2H), 2.44–1.28 (m, 23H); MS (APCI): m/z 587 $[M+1]^+$; Anal. calcd for $C_{33}H_{42}N_6O_4$ (586.6): C 67.55, H 7.22, N 14.32, found: C 67.88, H 7.66, N 14.57; $R_f=0.60$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (14:2:84)]; HPLC: $t_R=5.92$ min, purity: 92.8% [$CH_3CN/H_2O/TFA$ (50:50:0.1%)].

General method for the synthesis of L-pAad-L-His(1,2-di-alkyl)-L-ProNH₂ (16a–m)

The dipeptide salt (**13a–m**, 1 mmol) was neutralized by addition of a solution of 7 N NH₃ in CH₃OH (10 mL) for 10 min at 0 °C. A solution of free peptide in DMF (4 mL) was cooled to 4 °C, and pAad-OPfp (**15**, 0.81 mmol) was added, and the reaction mixture was stirred at 4 °C for 36 h. The solvent was removed under reduced pressure, and the resulting residue was purified by column chromatography over neutral alumina using CH₃OH/CHCl₃ (2:8) as eluent to afford tripeptides **16a–m**.

L-pAad-L-His(1-benzyl-2-propyl)-L-ProNH₂ (16a). Yield: 26%; mp: 74–76 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.31$ (m, 3H), 7.16 (m, 2H), 6.93 (s, 1H), 5.12 (s, 2H), 4.79 (m, 1H), 4.40 (m, 1H), 4.00 (m, 1H), 3.81 (m, 1H), 3.44 (m, 1H), 2.95 (m, 2H), 2.59 (t, 2H, $J=7.5$ Hz), 2.29–1.66 (m, 10H), 1.58 (m, 2H, CH₂), 0.89 (t, 3H, $J=7.2$ Hz); MS (APCI): m/z 509 $[M+1]^+$; Anal. calcd for $C_{27}H_{36}N_6O_4$ (508.6): C 63.76, H 7.13, N 16.52, found: C 64.07, H 7.31, N 16.19; $R_f=0.48$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (16:2:82)]; HPLC: $t_R=4.71$ min, purity: 99.8% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

L-pAad-L-His(1-benzyl-2-isopropyl)-L-ProNH₂ (16b). Yield: 13%; mp: 68–70 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.31$ (m, 3H), 7.14 (m, 2H), 6.92 (s, 1H), 5.15 (s, 2H), 4.82 (m, 1H), 4.38 (m, 1H), 4.01 (m, 1H), 3.79 (m, 1H), 3.45 (m, 1H), 3.04 (m, 1H), 2.97 (m, 2H), 2.29–1.68 (m, 10H), 1.18 (m, 6H); MS (APCI): m/z 509 $[M+1]^+$; Anal. calcd for $C_{27}H_{36}N_6O_4$ (508.6): C 63.76, H 7.13, N 16.52, found: C 64.07, H 7.31, N 16.19; $R_f=0.46$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (16:2:82)]; HPLC: $t_R=4.63$ min, purity: 95.9% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

L-pAad-L-His(1-benzyl-2-tert-butyl)-L-ProNH₂ (16c). Yield: 19%; mp: 77–80 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.29$ (m, 3H), 7.07 (m, 2H), 6.81 (s, 1H), 5.36 (s, 2H), 4.81 (m, 1H), 4.39 (m, 1H), 4.00 (m, 1H), 3.81 (m, 1H), 3.58 (m, 1H), 2.96 (m, 2H), 2.30–1.70 (m, 10H), 1.37 (s, 9H); MS (APCI): m/z 523 $[M+1]^+$; Anal. calcd for $C_{28}H_{38}N_6O_4$ (522.6): C 64.35, H 7.33, N 16.08, found: C 64.46, H 7.55, N 15.79; $R_f=0.42$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (16:2:82)]; HPLC: $t_R=4.82$ min, purity: 98.9% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

L-pAad-L-His(1-benzyl-2-cyclopropyl)-L-ProNH₂ (16d). Yield: 24%; mp: 72–74 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.27$ (m, 3H), 7.11 (m, 2H), 6.94 (s, 1H), 5.22 (s, 2H), 4.77 (m, 1H), 4.37 (m, 1H), 3.99 (m, 1H), 3.75 (m, 1H), 3.43 (m, 1H), 2.92 (m, 2H), 2.28–1.67 (m, 11H), 0.88 (m, 4H); MS (APCI): m/z 507 $[M+1]^+$; Anal. calcd for $C_{27}H_{34}N_6O_4$ (506.6): C 64.01, H 6.76, N 16.59, found: C 63.96, H 6.64,

N 16.51; $R_f=0.54$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (16:2:82)]; HPLC: $t_R=4.51$ min, purity: 96.6% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

L-pAad-L-His(1-benzyl-2-cyclopentyl)-L-ProNH₂ (16e). Yield: 28%; mp: 80–83 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.31$ (m, 3H), 7.14 (m, 2H), 6.93 (s, 1H), 4.88 (s, 2H), 4.81 (m, 1H), 4.38 (m, 1H), 4.01 (m, 1H), 3.75 (m, 1H), 3.43 (m, 1H), 3.05 (m, 1H), 2.96 (m, 2H), 2.28–1.60 (m, 18H); MS (APCI): m/z 535 $[M+1]^+$; Anal. calcd for $C_{29}H_{38}N_6O_4$ (534.7): C 65.15, H 7.16, N 15.72, found: C 65.34, H 6.94, N 16.07; $R_f=0.58$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (16:2:82)]; HPLC: $t_R=5.39$ min, purity: 97.8% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

L-pAad-L-His(1-benzyl-2-cyclohexyl)-L-ProNH₂ (16f). Yield: 23%; mp: 88–90 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.31$ (m, 3H), 7.16 (m, 2H), 6.92 (s, 1H), 5.14 (s, 2H), 4.81 (m, 1H), 4.38 (m, 1H), 4.01 (m, 1H), 3.78 (m, 1H), 3.45 (m, 1H), 2.96 (m, 2H), 2.82 (m, 1H, CH), 2.29–1.28 (m, 20H); MS (APCI): m/z 549 $[M+1]^+$; Anal. calcd for $C_{30}H_{40}N_6O_4$ (548.7): C 65.67, H 7.35, N 15.32, found: C 65.98, H 7.23, N 14.97; $R_f=0.62$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (16:2:82)]; HPLC: $t_R=6.32$ min, purity: 93.1% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

L-pAad-L-His[1-benzyl-2-(adamantan-1-yl)]-L-ProNH₂ (16g). Yield: 17%; mp: 96–98 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.29$ (m, 3H), 7.02 (m, 2H), 6.76 (s, 1H), 5.37 (m, 2H), 4.71 (m, 1H), 4.38 (m, 1H), 3.99 (m, 1H), 3.80 (m, 1H), 3.58 (m, 1H), 2.94 (m, 2H), 2.27 (m, 4H), 2.14–1.22 (m, 21H); MS (APCI): m/z 601 $[M+1]^+$; Anal. calcd for $C_{34}H_{44}N_6O_4$ (600.3): C 67.98, H 7.38, N 13.99, found: C 68.25, H 7.18, N 13.50; $R_f=0.52$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (8:2:90)]; HPLC: $t_R=9.49$ min, purity: 94.4% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

L-pAad-L-His(1-methyl-2-propyl)-L-ProNH₂ (16h). Yield: 32%; mp: 64–66 °C (dec.); ¹H NMR (CDCl₃): $\delta=8.19$ (bs, 1H), 6.63 (s, 1H), 4.77 (m, 1H), 4.50 (m, 1H), 4.02 (m, 1H), 3.67 (m, 1H), 3.52 (s, 3H), 3.21 (m, 1H), 2.97 (m, 2H), 2.53 (t, 2H, $J=7.5$ Hz), 2.42–1.72 (m, 10H), 1.58 (m, 2H, CH₂), 0.90 (t, 3H, $J=7.2$ Hz); MS (APCI): m/z 433 $[M+1]^+$; Anal. calcd for $C_{21}H_{33}N_6O_4$ (432.5): C 58.32, H 7.46, N 19.43, found: C 58.64, H 7.57, N 19.11; $R_f=0.55$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (20:2:78)]; HPLC: $t_R=4.93$ min, purity: 94.9% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

L-pAad-L-His(1-methyl-2-isopropyl)-L-ProNH₂ (16i). Yield: 38%; mp: 70–71 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.16$ (s, 1H), 4.96 (m, 1H), 4.45 (m, 1H), 4.05 (m, 1H), 3.81 (m, 1H), 3.77 (s, 3H), 3.56 (m, 1H), 3.37 (m, 1H), 3.11 (m, 2H), 2.28 (m, 4H), 2.09–1.72 (m, 6H), 1.38 (m, 6H); MS (APCI): m/z 433 $[M+1]^+$; Anal. calcd for $C_{21}H_{32}N_6O_4$ (432.5): C 58.32, H 7.46, N 19.43, found: C 58.44, H 7.59, N 19.58; $R_f=0.55$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (20:2:78)]; HPLC: $t_R=4.85$ min, purity: 95.5% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

L-pAad-L-His(1-methyl-2-tert-butyl)-L-ProNH₂ (16j). Yield: 38%; mp: 74–75 °C (dec.); ¹H NMR (CD₃OD): $\delta=7.20$ (s, 1H), 5.00 (m, 1H), 4.43 (m, 1H), 4.05 (m, 1H), 3.93 (s, 3H), 3.77 (m, 1H), 3.59 (m, 1H), 3.09 (m, 2H), 2.45–1.72 (m, 10H), 1.52 (s, 9H); MS (APCI): m/z 447 $[M+1]^+$; Anal. calcd for $C_{22}H_{34}N_6O_4$ (446.5): C 59.17, H 7.67, N 18.82, found: C 58.99, H 7.53, N 19.11; $R_f=0.57$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (20:2:78)]; HPLC: $t_R=5.42$ min, purity: 99.2% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

L-pAad-L-His(1-methyl-2-cyclopentyl)-L-ProNH₂ (16k). Yield: 27%; mp: 77–79 °C (dec.); ¹H NMR (CD₃OD): $\delta=6.83$ (s, 1H), 4.82 (m, 1H), 4.38 (m, 1H), 4.02 (m, 1H), 3.81 (m, 1H), 3.59 (s, 3H), 3.55 (m, 1H), 3.17 (m, 1H), 2.93 (m, 2H), 2.27–1.28 (m, 18H); MS (APCI): m/z 459 $[M+1]^+$; Anal. calcd for $C_{23}H_{34}N_6O_4$ (458.7): C 60.24, H 7.47, N 18.33, found: C 60.22, H 7.42, N 18.31; $R_f=0.60$ [$CH_3OH/25\%$

NH₄OH/CHCl₃ (20:2:78)]; HPLC: t_R = 6.98 min, purity: 96.5% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pAad-L-His(1-methyl-2-cyclohexyl)-L-ProNH₂ (16i): Yield: 22%; mp: 82–84 °C (dec.); ¹H NMR (CD₃OD): δ = 6.77 (s, 1H), 4.80 (m, 1H), 4.36 (m, 1H), 4.02 (m, 1H), 3.81 (m, 1H), 3.57 (s, 3H), 3.53 (m, 1H), 2.92 (m, 2H), 2.73 (m, 1H), 2.30–1.26 (m, 20H); MS (APCI): m/z 473 [M+1]⁺; Anal. calcd for C₂₄H₃₆N₆O₄ (472.7): C 61.00, H 7.68, N 17.78, found: C 61.15, H 7.98, N 17.47; R_f = 0.62 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: t_R = 8.95 min, purity: 97.8% [CH₃CN/H₂O/TFA (20:80:0.1%)].

L-pAad-L-His[1-methyl-2-(adamantan-1-yl)]-L-ProNH₂ (16m): Yield: 15%; mp: 88–90 °C (dec.); ¹H NMR (CDCl₃): δ = 6.61 (s, 1H), 4.83 (m, 1H), 4.46 (m, 1H), 4.00 (m, 1H), 3.73 (s, 3H), 3.66 (m, 1H), 3.31 (m, 1H), 2.91 (m, 2H), 2.35 (m, 4H), 2.18–1.25 (m, 21H); MS (APCI): m/z 525 [M+1]⁺; Anal. calcd for C₂₈H₄₀N₆O₄ (524.6): C 64.10, H 7.68, N 16.02, found: C 63.92, H 7.96, N 15.73; R_f = 0.59 [CH₃OH/25% NH₄OH/CHCl₃ (20:2:78)]; HPLC: t_R = 24.78 min, purity: 94.7% [CH₃CN/H₂O/TFA (20:80:0.1%)].

Synthesis of (1R)-3-Ocp-L-His(1-alkyl)-L-ProNH₂ (21a–e)

The dipeptide salt (**19a–e**, 1 mmol) was neutralized with a solution of 7 N NH₃ in CH₃OH (10 mL) for 10 at 0 °C min. HOBt (1.1 mmol) was added to a solution of free dipeptide in anhydrous DMF (10 mL). The reaction mixture was cooled to –10 °C, and DIC (1.1 mmol) was added in one portion. The reaction mixture was stirred for an additional 5 min at –10 °C. (1R)-3-Ocp-OH (**20**, 1 mmol) was then added to the reaction mixture, and stirring was continued for an additional 36 h at 4 °C. The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography on neutral alumina using CH₃OH/CHCl₃ (10:90) as eluent to afford tripeptides **21a–e**.

(1R)-3-Ocp-L-His(1-methyl)-L-ProNH₂ (21a): Yield: 30%; mp: 101–103 °C (dec.); ¹H NMR (CD₃OD): δ = 7.50 (s, 1H), 6.94 (s, 1H), 4.79 (m, 1H), 4.39 (m, 1H), 3.72 (m, 1H), 3.67 (s, 3H), 3.43 (m, 1H), 3.05 (m, 1H), 2.96 (m, 2H), 2.35–1.91 (m, 10H); ¹³C NMR (CD₃OD): δ = 24.57, 25.84, 29.38, 31.97, 33.02, 37.57, 40.16, 42.36, 46.81, 50.51, 60.20, 117.33, 137.31, 139.77, 169.40, 174.84, 177.59, 216.11; MS (APCI): m/z 376 [M+1]⁺; Anal. calcd for C₁₈H₂₅N₅O₄ (375.4): C 57.59, H 6.71, N 18.65, found: C 57.97, H 6.45, N 18.51; R_f = 0.44 [CH₃OH/25% NH₄OH/CHCl₃ (12:2:86)]; HPLC: t_R = 3.98 min, purity: 99.5% [CH₃CN/H₂O/TFA (20:80:0.1%)].

(1R)-3-Ocp-L-His(1-ethyl)-L-ProNH₂ (21b): Yield: 49%; mp: 96–98 °C (dec.); ¹H NMR (CD₃OD): δ = 7.58 (s, 1H), 7.03 (s, 1H), 4.80 (m, 1H), 4.39 (m, 1H), 4.00 (q, 2H, J = 7.2 Hz), 3.75 (m, 1H), 3.38 (m, 1H), 3.06 (m, 1H), 2.96 (m, 2H), 2.34–1.90 (m, 10H), 1.40 (t, 3H, J = 7.2 Hz); ¹³C NMR (CD₃OD): δ = 15.72, 24.10, 26.20, 29.70, 31.67, 38.10, 39.95, 41.34, 42.70, 46.60, 50.99, 60.45, 115.35, 135.84, 138.74, 169.27, 174.0, 177.10, 215.87; MS (APCI): m/z 390 [M+1]⁺; Anal. calcd for C₁₉H₂₇N₅O₄ (389.5): C 58.60, H 6.99, N 17.98, found: C 58.91, H 7.07, N 17.61; R_f = 0.48 [CH₃OH/25% NH₄OH/CHCl₃ (12:2:86)]; HPLC: t_R = 4.30 min, purity: 97.4% [CH₃CN/H₂O/TFA (20:80:0.1%)].

(1R)-3-Ocp-L-His(1-propyl)-L-ProNH₂ (21c): Yield: 31%; mp: 90–92 °C (dec.); ¹H NMR (CD₃OD): δ = 7.56 (s, 1H), 7.01 (s, 1H), 4.78 (m, 1H), 4.40 (m, 1H), 3.92 (t, 2H, J = 6.6 Hz), 3.78 (m, 1H), 3.40 (m, 1H), 3.06 (m, 1H), 2.96 (m, 2H), 2.35–1.95 (m, 10H), 1.79 (m, 2H), 0.89 (t, 3H, J = 6.9 Hz); ¹³C NMR (CD₃OD): δ = 11.05, 23.64, 24.08, 25.94, 29.08, 32.10, 37.12, 40.44, 42.97, 46.50, 48.24, 51.01, 60.20,

116.19, 136.01, 139.60, 168.86, 174.53, 177.07, 216.50; MS (APCI): m/z 404 [M+1]⁺; Anal. calcd for C₂₀H₂₉N₅O₄ (403.5): C 59.54, H 7.24, N 17.36, found: C 59.83, H 7.49, N 17.39; R_f = 0.55 [CH₃OH/25% NH₄OH/CHCl₃ (12:2:86)]; HPLC: t_R = 4.95 min, purity: 99.6% [CH₃CN/H₂O/TFA (20:80:0.1%)].

(1R)-3-Ocp-L-His(1-isopropyl)-L-ProNH₂ (21d): Yield: 20%; mp: 88–90 °C (dec.); ¹H NMR (CD₃OD): δ = 7.64 (s, 1H), 7.11 (s, 1H), 4.80 (m, 1H), 4.40 (m, 1H), 3.77 (m, 1H), 3.36 (m, 1H), 3.04 (m, 1H), 2.96 (m, 3H), 2.35–1.95 (m, 10H), 1.46 (d, 6H, J = 6.0 Hz); ¹³C NMR (CD₃OD): δ = 24.33, 25.84, 26.46, 29.38, 33.09, 37.57, 40.16, 42.36, 46.81, 47.95, 51.08, 60.20, 112.02, 133.40, 137.63, 169.49, 174.84, 177.84, 177.51, 216.25; MS (APCI): m/z 404 [M+1]⁺; Anal. calcd for C₂₀H₂₉N₅O₄ (403.5): C 59.54, H 7.24, N 17.36, found: C 59.68, H 6.96, N 17.74; R_f = 0.53 [CH₃OH/25% NH₄OH/CHCl₃ (12:2:86)]; HPLC: t_R = 4.79 min, purity: 97.6% [CH₃CN/H₂O/TFA (20:80:0.1%)].

(1R)-3-Ocp-L-His(1-benzyl)-L-ProNH₂ (21e): Yield: 18%; mp: 96–98 °C (dec.); ¹H NMR (CDCl₃): 7.71 (s, 1H), 7.35 (m, 5H), 7.05 (s, 1H), 5.19 (m, 2H), 4.80 (m, 1H), 4.44 (m, 1H), 3.79 (m, 1H), 3.35 (m, 1H), 3.07 (m, 1H), 2.96 (m, 2H), 2.35–1.71 (m, 10H); ¹³C NMR (CD₃OD): δ = 23.87, 24.84, 29.38, 32.00, 37.30, 39.95, 42.05, 42.81, 50.10, 50.94, 60.59, 117.19, 127.13, 128.20, 128.99, 136.18, 137.63, 139.05, 169.27, 174.57, 177.59, 216.05; MS (APCI): m/z 452 [M+1]⁺; Anal. calcd for C₂₄H₂₉N₅O₄ (451.5): C 63.84, H 6.47, N 15.51, found: C 64.06, H 6.20, N 15.65; R_f = 0.59 [CH₃OH/25% NH₄OH/CHCl₃ (12:2:86)]; HPLC: t_R = 8.43 min, purity: 97.0% [CH₃CN/H₂O/TFA (20:80:0.1%)].

General method for the synthesis of (1R)-3-Ocp-L-His(1,2-di-alkyl)-L-ProNH₂ (22a–l)

Protected dipeptide (**12**, 0.73 mmol) was treated with a solution of 40% TFA in CH₂Cl₂ (15 mL) at 4 °C for 30 min. The salt was neutralized with a solution of 7 N NH₃ in CH₃OH (10 mL) for 10 min at 0 °C. HOBt (1.1 mmol) was added to a solution of free dipeptides in anhydrous DMF (10 mL). The reaction mixture was cooled to –10 °C, and DIC (1.1 mmol) was added in one portion. The reaction mixture was stirred for an additional 5 min at –10 °C. (1R)-3-Ocp-OH (**20**, 1 mmol) was then added to the reaction mixture, and stirring was continued for an additional 36 h at 4 °C. The solvent was removed, and the crude product was purified by column chromatography on neutral alumina using CH₃OH/CHCl₃ (10:90) to afford tripeptides **22a–l**.

(1R)-3-Ocp-L-His(1-benzyl-2-propyl)-L-ProNH₂ (22a): Yield: 27%; mp: 110–112 °C (dec.); ¹H NMR (CD₃OD): δ = 7.31 (m, 3H), 7.17 (m, 2H), 6.93 (s, 1H), 5.13 (s, 2H), 4.81 (m, 1H), 4.39 (m, 1H), 3.78 (m, 1H), 3.46 (m, 1H), 3.03 (m, 1H), 2.95 (m, 2H), 2.61 (t, 2H, J = 7.8 Hz), 2.34–1.93 (m, 10H), 1.57 (m, 2H), 0.89 (t, 3H, J = 7.2 Hz); ¹³C NMR (CD₃OD): δ = 14.44, 21.69, 24.33, 25.84, 29.38, 29.88, 32.20, 37.57, 40.16, 42.36, 46.81, 48.60, 51.06, 60.20, 117.97, 127.15, 128.82, 128.20, 135.48, 135.65, 148.95, 169.28, 174.64, 177.59, 216.11; MS (APCI): m/z 494 [M+1]⁺; Anal. calcd for C₂₇H₃₅N₅O₄ (493.6): C 65.70, H 7.15, N 14.19, found: 65.94, H 7.29, N 14.28; R_f = 0.52 [CH₃OH/25% NH₄OH/CHCl₃ (12:2:86)]; HPLC: t_R = 5.14 min, purity: 99.0% [CH₃CN/H₂O/TFA (40:60:0.1%)].

(1R)-3-Ocp-L-His(1-benzyl-2-isopropyl)-L-ProNH₂ (22b): Yield: 24%; mp: 120–122 °C (dec.); ¹H NMR (CD₃OD): δ = 7.30 (m, 3H), 7.16 (m, 2H), 6.90 (s, 1H), 5.15 (s, 2H), 4.83 (m, 1H), 4.38 (m, 1H), 3.81 (m, 1H), 3.51 (m, 1H), 3.05 (m, 2H), 2.95 (m, 2H), 2.34–1.93 (m, 10H), 1.17 (d, 6H, $2 \times J$ = 6.9 Hz); ¹³C NMR (CD₃OD): δ = 22.20, 24.17, 25.80, 28.50, 29.31, 33.19, 37.54, 40.10, 42.48, 46.98, 49.35, 51.13,

60.60, 116.20, 127.06, 128.43, 128.50, 134.14, 135.05, 153.87, 169.50, 174.37, 177.55, 215.13; MS (APCI): m/z 494 $[M+1]^+$; Anal. calcd for $C_{27}H_{35}N_5O_4$ (493.6): C 65.70, H 7.15, N 14.19, found: 65.79, H 7.38, N 14.23; $R_f=0.50$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (10:2:88)]; HPLC: $t_R=4.95$ min, purity: 99.4% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

(1R)-3-Ocp-L-His(1-benzyl-2-tert-butyl)-L-ProNH₂ (22c). Yield: 10%; mp: 126–128 °C (dec.); ¹H NMR (CD_3OD): $\delta=7.30$ (m, 3H), 7.08 (m, 2H), 6.90 (s, 1H), 5.36 (m, 2H), 4.81 (m, 1H), 4.39 (m, 1H), 3.79 (m, 1H), 3.56 (m, 1H), 3.03 (m, 1H), 2.94 (m, 2H), 2.35–1.95 (m, 10H), 1.38 (s, 9H); ¹³C NMR (CD_3OD): $\delta=24.15, 25.89, 28.28, 29.34, 32.05, 32.86, 37.57, 40.10, 43.36, 46.70, 50.34, 51.20, 60.16, 115.90, 126.96, 128.13, 128.76, 135.00, 135.83, 154.98, 169.49, 174.52, 177.51, 216.15$; MS (APCI): m/z 508 $[M+1]^+$; Anal. calcd for $C_{28}H_{35}N_5O_4$ (507.6): C 66.25, H 7.35, N 13.80, found: 66.36, H 7.04, N 14.17; $R_f=0.56$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (10:2:88)]; HPLC: $t_R=5.26$ min, purity: 95.5% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

(1R)-3-Ocp-L-His(1-benzyl-2-cyclopropyl)-L-ProNH₂ (22d). Yield: 29%; mp: 116–118 °C (dec.); ¹H NMR (CD_3OD): $\delta=7.32$ (m, 3H), 7.21 (m, 2H), 6.83 (s, 1H), 5.23 (s, 2H), 4.77 (m, 1H), 4.36 (m, 1H), 3.76 (m, 1H), 3.44 (m, 1H), 3.00 (m, 1H), 2.92 (m, 2H), 2.40–1.92 (m, 10H), 1.84 (m, 1H), 0.88 (m, 4H); ¹³C NMR (CD_3OD): $\delta=8.52, 8.87, 24.13, 25.80, 29.30, 32.10, 37.26, 40.19, 42.40, 46.81, 49.57, 51.11, 60.17, 115.79, 127.34, 128.20, 128.82, 134.79, 136.59, 152.02, 169.54, 174.64, 177.77, 216.30$; MS (APCI): m/z 492 $[M+1]^+$; Anal. calcd for $C_{27}H_{33}N_5O_4$ (491.6): C 65.97, H 6.77, N 14.25, found: C 66.38, H 7.16, N 13.85; $R_f=0.53$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (10:2:88)]; HPLC: $t_R=4.72$ min, purity: 96.0% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

(1R)-3-Ocp-L-His(1-benzyl-2-cyclopentyl)-L-ProNH₂ (22e). Yield: 23%; mp: 122–124 °C (dec.); ¹H NMR (CD_3OD): $\delta=7.31$ (m, 3H), 7.15 (m, 2H), 6.91 (s, 1H), 5.14 (s, 2H), 4.71 (m, 1H), 4.36 (m, 1H), 3.81 (m, 1H), 3.50 (m, 1H), 3.06 (m, 2H), 2.96 (m, 2H), 2.34–1.60 (m, 18H); ¹³C NMR (CD_3OD): $\delta=24.78, 25.84, 29.38, 31.82, 37.50, 39.15, 40.16, 42.36, 46.71, 48.88, 51.17, 60.32, 116.44, 127.13, 128.43, 128.65, 134.41, 135.17, 150.33, 169.50, 174.64, 177.58, 214.13$; MS (APCI): m/z 520 $[M+1]^+$; Anal. calcd for $C_{29}H_{37}N_5O_4$ (519.6): C 67.03, H 7.18, N 13.48, found: C 67.50, H 7.47, N 13.03; $R_f=0.57$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (10:2:88)]; HPLC: $t_R=5.98$ min, purity: 93.0% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

(1R)-3-Ocp-L-His(1-benzyl-2-cyclohexyl)-L-ProNH₂ (22f). Yield: 24%; mp: 130–132 °C (dec.); ¹H NMR (CD_3OD): $\delta=7.31$ (m, 3H), 7.16 (m, 2H), 6.90 (s, 1H), 5.14 (s, 2H), 4.85 (m, 1H), 4.37 (m, 1H), 3.80 (m, 1H), 3.47 (m, 1H), 3.02 (m, 1H), 2.94 (m, 2H), 2.70 (m, 1H), 2.34–1.28 (m, 20H); ¹³C NMR (CD_3OD): $\delta=24.30, 25.76, 26.13, 27.03, 29.13, 32.01, 32.23, 35.14, 37.57, 39.96, 42.12, 46.81, 49.08, 51.04, 60.03, 116.36, 127.13, 128.20, 128.82, 134.57, 135.29, 150.08, 169.34, 174.64, 177.59, 216.12$; MS (APCI): m/z 534 $[M+1]^+$; Anal. calcd for $C_{30}H_{39}N_5O_4$ (533.7): C 67.52, H 7.37, N 13.12, found: C 67.64, H 7.50, N 13.38; $R_f=0.58$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (10:2:88)]; HPLC: $t_R=7.27$ min, purity: 99.3% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

(1R)-3-Ocp-L-His[1-benzyl-2-(adamantan-1-yl)]-L-ProNH₂ (22g). Yield: 18%; mp: 136–138 °C (dec.); ¹H NMR (CD_3OD): $\delta=7.38$ (m, 3H), 7.15 (m, 2H), 7.04 (s, 1H), 5.56 (s, 2H), 4.97 (m, 1H), 4.42 (m, 1H), 3.72 (m, 1H), 3.59 (m, 1H), 3.06 (m, 2H), 3.00 (m, 1H), 2.33–1.28 (m, 25H); ¹³C NMR (CD_3OD): $\delta=24.34, 25.84, 29.31, 29.38, 32.16, 35.99, 37.44, 40.16, 40.96, 42.36, 46.65, 50.35, 51.26, 59.12, 115.87, 127.05, 128.76, 135.04, 136.55, 151.17, 169.49, 174.12, 177.14, 215.80$; MS (APCI): m/z 586 $[M+1]^+$; Anal. calcd for $C_{34}H_{43}N_5O_4$ (585.7): C 69.72, H 7.40, N 11.96, found: C 69.38, H 7.69,

N 12.29; $R_f=0.62$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (10:2:88)]; HPLC: $t_R=11.07$ min, purity: 98.3% [$CH_3CN/H_2O/TFA$ (40:60:0.1%)].

(1R)-3-Ocp-L-His(1-methyl-2-propyl)-L-ProNH₂ (22h). Yield: 28%; mp: 100–103 °C (dec.); ¹H NMR (CD_3OD): $\delta=6.81$ (s, 1H), 4.77 (m, 1H), 4.39 (m, 1H), 3.78 (m, 1H), 3.57 (s, 3H), 3.45 (m, 1H), 3.08 (m, 1H), 2.96 (m, 2H), 2.64 (t, 2H, $J=7.5$ Hz), 2.35–1.94 (m, 10H), 1.67 (m, 2H), 0.96 (t, 3H, $J=7.2$ Hz); MS (APCI): m/z 418 $[M+1]^+$; Anal. calcd for $C_{21}H_{31}N_5O_4$ (417.5): C 60.41, H 7.48, N 16.77, found: C 60.52, H 7.61, N 17.10; $R_f=0.43$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (12:2:86)]; HPLC: $t_R=5.46$ min, purity: 98.4% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

(1R)-3-Ocp-L-His(1-methyl-2-isopropyl)-L-ProNH₂ (22i). Yield: 31%; mp: 96–98 °C (dec.); ¹H NMR (CD_3OD): $\delta=6.80$ (s, 1H), 4.80 (m, 1H), 4.39 (m, 1H), 3.79 (m, 1H), 3.59 (s, 3H), 3.50 (m, 1H), 3.11 (m, 2H), 2.93 (m, 2H), 2.36–1.93 (m, 10H), 1.28 (m, 6H); MS (APCI): m/z 418 $[M+1]^+$; Anal. calcd for $C_{21}H_{31}N_5O_4$ (417.5): C 60.41, H 7.48, N 16.77, found: C 60.25, H 7.37, N 16.61; $R_f=0.42$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (12:2:86)]; HPLC: $t_R=5.17$ min, purity: 98.3% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

(1R)-3-Ocp-L-His(1-methyl-2-tert-butyl)-L-ProNH₂ (22j). Yield: 35%; mp: 104–106 °C (dec.); ¹H NMR (CD_3OD): $\delta=6.78$ (s, 1H), 4.79 (m, 1H), 4.37 (m, 1H), 3.78 (m, 1H), 3.73 (s, 3H), 3.53 (m, 1H), 3.07 (m, 1H), 2.89 (m, 2H), 2.36–1.89 (m, 10H), 1.40 (s, 9H, $3 \times CH_3$); MS (APCI): m/z 432 $[M+1]^+$; Anal. calcd for $C_{22}H_{33}N_5O_4$ (431.5): C 61.23, H 7.71, N 16.23; C 60.99, H 8.09, N 15.97; $R_f=0.46$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (12:2:86)]; HPLC: $t_R=5.88$ min, purity: 99.7% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

(1R)-3-Ocp-L-His(1-methyl-2-cyclohexyl)-L-ProNH₂ (22k). Yield: 19%; mp: 110–112 °C (dec.); ¹H NMR (CD_3OD): $\delta=6.78$ (s, 1H), 4.80 (m, 1H), 4.38 (m, 1H), 3.79 (m, 1H), 3.57 (s, 3H), 3.47 (m, 1H), 3.08 (m, 1H), 2.90 (m, 2H), 2.74 (m, 1H), 2.35–1.28 (m, 20H); MS (APCI): m/z 458 $[M+1]^+$; Anal. calcd for $C_{24}H_{35}N_5O_4$ (457.6): C 63.00, H 7.71, N 15.31, found: C 63.08, H 7.78, N 15.36; $R_f=0.54$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (12:2:86)]; HPLC: $t_R=10.45$ min, purity: 96.6% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

(1R)-3-Ocp-L-His[1-methyl-2-(adamantan-1-yl)]-L-ProNH₂ (22l). Yield: 25%; mp: 118–120 °C (dec.); ¹H NMR (CD_3OD): $\delta=6.75$ (s, 1H), 4.79 (m, 1H), 4.37 (m, 1H), 3.79 (m, 1H), 3.77 (s, 3H), 3.54 (m, 1H), 3.08 (m, 1H), 2.89 (m, 2H), 2.34 (m, 2H), 2.29–1.81 (m, 23H); MS (APCI): m/z 510 $[M+1]^+$; Anal. calcd for $C_{28}H_{39}N_5O_4$ (509.6): C 65.99, H 7.71, N 13.74, found: C 66.28, H 8.05, N 13.35; $R_f=0.59$ [$CH_3OH/25\% NH_4OH/CHCl_3$ (12:2:86)]; HPLC: $t_R=26.14$ min, purity: 95.1% [$CH_3CN/H_2O/TFA$ (20:80:0.1%)].

Receptor binding assays

Receptor binding studies were carried out according to a procedure described previously.^[36] Briefly, HEK293EM cells stably expressing either TRH-R1 or TRH-R2 were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 200 mg mL⁻¹ hygromycin. For equilibrium binding experiments, cells were seeded into 24-well plates (1.5 × 10⁵ cells per well). After 48 h, cells were incubated at 37 °C for 1 h with [³H][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 nonlinear regression analysis and drawn with the Prism version 3 software package (GraphPad Software, Inc.) using the formula $K_i = (IC_{50}) / \{1 + ([L]/K_d)\}$, in which

IC_{50} is the concentration of unlabeled analogue that half-competes and K_d is the equilibrium dissociation constant for [3H][N(1)-Me-His]TRH.

Luciferase activity assays

On the day prior to transfection, cells stably expressing either TRH-R1 or TRH-R2 were seeded into 24-well plates (1.5×10^5 cells per well). After 16 h, the medium was aspirated, and the cells (~50% confluent) were co-transfected with plasmid DNA encoding CREB and the CREB-activated luciferase gene (PathDetect CREB trans-Reporting SystemTM, 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, and 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 was measured for 10 s in a TR717 Microplate luminometer (Tropix, Bedford, MA, USA). The luciferase activity levels detected by this assay reflect the activation of signaling by TRH analogues.

Analeptic activity

Male Swiss albino mice (20–30 g) were procured from the Central Animal Facility (CAF), NIPER, S.A.S. Nagar. Animals were housed in a room at $22 \pm 2^\circ C$ with 12 h light/dark cycles and were allowed free access to pellet food and water. All procedures used in this study were approved by the Institutional Animal Ethics Committee (IAEC), NIPER (experimental protocol approval numbers IAEC/08/43 and IAEC/09/01) and were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

The analeptic activities of TRH analogues were studied by the antagonism of pentobarbital-induced sleep time in albino Swiss mice as described elsewhere.^[40] Test compounds were dissolved in vehicle (saline). Vehicle (5 mL kg^{-1}) or TRH/TRH analogues ($10 \mu\text{mol kg}^{-1}$, equivalent to 3.7 mg kg^{-1} TRH) were injected via the tail vein. Ten minutes after treatment, each animal received sodium pentobarbital 50 mg kg^{-1} i.p. The sleeping time was recorded as the time elapsed from the onset of loss of righting reflex until it returned. The sleep time in treatment groups was compared with control group by one-way analysis of variance (ANOVA) followed by post hoc Tukey test at $p < 0.05$. Decrease in sleep time was calculated with Equation (1):

$$\% \text{ decrease in sleep time} = (1 - S/C) \times 100 \quad (1)$$

for which S = sleep time in compound-treated group, and C = sleeping time in vehicle-treated group.

Anticonvulsant activity

Animal seizure tests included one chemically induced seizure episode test and one for electrically induced seizures; these were conducted according to protocols described elsewhere.^[51,52] For the chemically induced convulsion test, administration of pentylenete-

trazole (PTZ, 65 mg kg^{-1} i.p.) was used to induce clonic seizures, reflecting a generalized seizure condition in humans. TRH/TRH analogues ($10 \mu\text{mol kg}^{-1}$ i.v.) were injected into mice ($n = 6-8$ per group) via the tail vein, 10 min prior to PTZ administration, whereas the control group was administered saline. The animals were observed for 10 min after PTZ challenge. Onset of, and latency to, generalized clonic convulsions with falling was recorded.

The electrical test employed was the maximal electric shock (MES) seizure test. The MES-induced seizure model was used to assess the potential of drugs against partial seizures. Seizures were induced in mice by applying a current of 45 mA through a corneal electrode for 0.2 s as described elsewhere with slight modification.^[52] Mice ($n = 6-8$ per group) were treated with saline or TRH ($10 \mu\text{mol kg}^{-1}$) or TRH analogues ($10 \mu\text{mol kg}^{-1}$ i.v.). Ten minutes later, seizures were induced by MES, and the mice were observed for duration of tonic hind limb extension in mice.

The Supporting Information contains detailed experimental procedures and spectral data for intermediates **12a–m**, **13a–m**, **18a–e**, and **19a–e**.

Acknowledgements

This work is supported by the Council of Scientific and Industrial Research, New Delhi (Grant No. 01(1861)/03/EMR-II). V.M. thanks the Department of Science and Technology, New Delhi (Grant No. SR/FTP/CS-79/2005), for the award of a young scientist fellowship. C.L.M. thanks the University Grant Commission for the award of a senior research fellowship.

Keywords: CNS · peptides · pharmacology · receptors · thyrotropin-releasing hormone

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Received: November 10, 2010

Revised: January 6, 2011

Published online on February 7, 2011