ELSEVIER

Il Farmaco 57 (2002) 479–486

IL FARMACO

www.elsevier.com/locate/farmac

Synthesis and biological evaluation of a novel pyroglutamyl-modified TRH analogue

L. Brunetti [a], I. Cacciatore [a], A. Di Stefano [a], S. Duprè [b], A. Giorgi [b], G. Luisi [a], B. Michelotto [a], G. Orlando [a], F. Pinnen [a,c,*], L. Recinella [a], P. Sozio [a], A. Spirito [b]

[a] *Dipartimento di Scienze del Farmaco, Università degli Studi 'G. D'Annunzio', Via dei Vestini 31, I-66013 Chieti, Italy*
[b] *Dipartimento di Scienze Biochimiche, Università degli Studi 'La Sapienza', P.le Aldo Moro 5, I-00185 Rome, Italy*
[c] *Centro di Studio per la Chimica del Farmaco del CNR, Università degli Studi 'La Sapienza', Rome, Italy*

Received 13 December 2001; accepted 17 February 2002

Abstract

The TRH analogue **3**, incorporating the (*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid (**1**) moiety in place of the native L-pyroglutamic acid (pGlu) residue, has been synthesized and fully characterized by ^{1}H and ^{13}C NMR. The effects of replacing pGlu with its sulphonamido counterpart on biological activity have been investigated. This peptide, which is significantly stabilized towards hydrolysis by pyroglutamyl peptidase type I (PP I, EC 3.4.19.3), has shown to maintain in vitro prolactin-releasing activity.

Keywords: Pyroglutamic acid; Pyroglutamyl peptidases; Sulphonamidopeptides; Synaptosomes; Thyrotropin-releasing hormone

1. Introduction

Distinctive biological activities of many peptides, such as their ability to recognize and bind to receptors and their relative stability towards inactivation by peptidases, are governed by specific structural modifications at the amino- and carboxy-terminal positions. The pyroglutamic acid (pGlu) residue is a frequent structural determinant in hormones and neuropeptides, where it is postulated to originate at the *N*-terminus from the post-translational cyclization of amino terminal glutamyl or glutaminyl residues; relevant physiological functions for this amino acid are suggested by the existence of enzymatic systems involved in its formation [1–4]. The pyroglutamate residue is hydrolytically removed from pyroglutamyl peptides and proteins by a unique class of enzymes, the pyroglutamyl peptidases (PPs) [5]; interestingly, two out of the three distinct PP forms identified to date in mammalian tissues show a substrate specificity restricted to the endocrine and CNS-active thyrotropin-releasing hormone (TRH: L-pyroglutamyl-L–histidyl–L-prolinamide, Fig. 1), and to TRH-like peptides.

Much interest is focused on these peptidases, since they are thought to play a role in regulating TRH availability to its receptors, as well as the extent and duration of its neuroendocrine activities. So far, a number of pyroglutamyl-modified TRH analogues have been synthesized in order to overcome susceptibility to PP degradation and consequently to prolong intrinsic activity [6–10]. It is worth noting that several of these

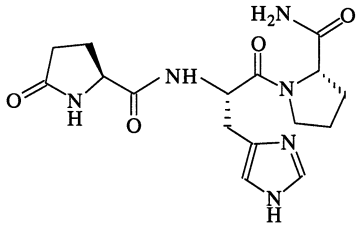


Fig. 1. TRH.

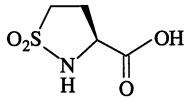
Abbreviations: Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature as given in *Eur. J. Biochem.* 138 (1984) 9–37. Additional abbreviations:; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*′-dimethylformamide; DMSO-d_6, hexadeuterated dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; MeOH, methanol; TEA, triethylamine; *Z*, benzyloxycarbonyl.

* Corresponding author.
E-mail address: fpinnen@unich.it (F. Pinnen).

0014-827X/02/$ - see front matter
PII: S0014-827X(02)01232-6

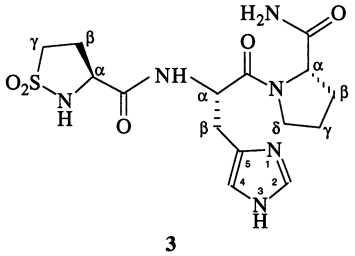
analogues were shown to possess enhanced CNS effects as a consequence of increased bioavailability [8,11].

In the course of our studies on the modification of bioactive peptides [12–15], we became interested in sulphonamidopeptides [16–18]. These pseudopeptides, characterized by an SO_2NH versus CONH replacement, contain a peptide bond surrogate which appears very attractive for more than one reason: stability toward enzymatic hydrolysis, H-bonding capacity, high polar character, and tetrahedral structure suitable to design transition state analogue protease inhibitors. In a preliminary communication, we reported the synthesis and some chemical properties of (*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid (**1**), the sulphonamido analogue of pyroglutamic acid [19].



1

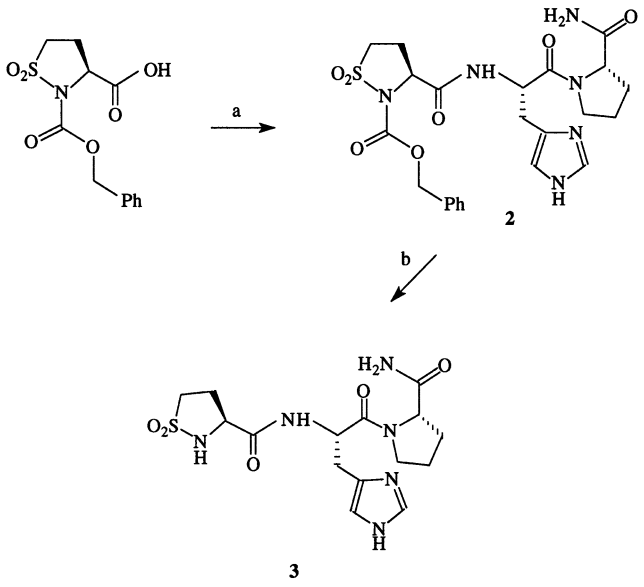
As an extension of our research in this field and taking into account the biological significance of the TRH/PP system, we were prompted to design a pyroglutamyl-modified TRH analogue containing the new γ-sultam **1**, with a view to achieving stabilization of the PP sensitive pGlu–His peptide bond as well as possible dissociation of the CNS from the hormonal activities. Here we report data on the synthesis, characterization and biological activity of the novel TRH analogue **3**.



3

2. Chemistry

In the synthesis of the TRH analogue **3**, still maintaining the central histidine residue of the parent peptide, we coped with some problems connected with the reactivities of both the γ-sultam and the imidazole rings. The TRH analogue **3** was synthesized using solution procedures by stepwise elongation of the peptide chain in the *C*-to-*N* direction as outlined in Scheme 1. Dipeptide amide H–His–Pro–NH_2 [20] was prepared by conventional DCC condensation of Boc–His–OH with H–Pro–NH_2 and subsequent acidolytic removal of the Boc protecting group. The modified amino acid (*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid (**1**) was synthesized according to the methods reported in our previous paper [19] and opportunely converted into its



Scheme 1. Synthesis of TRH analogue **3**. Reagents and conditions: (a) H–His–Pro–NH_2, isobutyl chloroformate, TEA, DMF, −10 °C, 20 min; 0 °C, 3 h; then r.t., 20 h; (b) H_2, Pd–C 10%, MeOH, r.t., 2 h.

N-benzyloxycarbonyl derivative for the subsequent coupling step. Direct condensation of *N*-unprotected **1** with H–His–Pro–NH_2 was found in fact to be inconvenient, owing to its low solubility in the solvents usually employed in coupling reactions, and in accordance with the well-known tendency of the SO_2NH group to undergo intra- and inter-molecular acylation reactions [21]. Coupling of (*S*)-2-benzyloxycarbonyl-isothiazolidine-1,1-dioxide-3-carboxylic acid with H–His–Pro–NH_2 was found to be difficult, due to the reactivity of the imidazo basic group. A variety of conditions were tried in order to overcome the problem and finally the precursor tripeptide was obtained in acceptable yields by using mixed anhydride activation. The resulting (*S*,*S*,*S*)-*N*-(2-benzyloxycarbonyl-isothiazolidine-1,1-dioxide-3-carbonyl)-histidyl-prolinamide (**2**) was then converted by catalytic hydrogenolysis with 10% Pd–C into the desired TRH analogue **3** without any side reactions and in good yields (Scheme 1). It should be noted here that, in accordance with the known low stability of sultams of type **1** under acidolytic conditions [22], a complex reaction mixture was obtained when HBr in acetic acid was used for the *N*-deprotection of **2**. The final peptide was purified by gel-permeation chromatography and fully characterized by NMR spectroscopy.

A correlation of the ^1H and ^{13}C NMR chemical shifts of the new sulphonamido analogue **3** with those of native TRH in DMSO-d_6 solution [23,24] is reported in Table 1. An analysis of spectral data indicates that **3** presents great conformational similarities to TRH. In particular, a *trans*/*cis* isomerism about the His–Pro peptide bond is observed for **3**. Measurement of the *cis*

content, made from relative peak intensities of C^{β} and C^{γ} atoms in DMSO-d_6 solution at room temperature [25], reveals a *cis*-configurated population of about 10–15%, comparable to that of TRH in the same solvent. The Pro C^{β} and C^{γ} atoms of **3** show prevailing resonances (δ 29.97 and 25.02 ppm, respectively) typical of *trans* Xaa–Pro bonds, which are accompanied by resonances at 31.83 and 22.89 δ ppm, typical of *cis* bonds [26]. Furthermore, as in the case of TRH, several resonances accompanied by satellite peaks, reflecting the *cis* content of the prolyl residue, are present in the ^{1}H NMR spectrum of compound **3** [23,25,27].

A characteristic spectral feature of **3** is connected with the preferred conformation of the histidyl residue. The observed $N^{\alpha}H–C^{\alpha}H$ coupling constant for histidine in DMSO-d_6 solution (7.5 Hz) is within the 7.2–8.0 Hz range which is reported for the same coupling in TRH [25,28] and in accordance with a preferentially extended backbone conformation. Furthermore, the values of His $C^{\alpha}H–C^{\beta}H$ vicinal coupling constants (6.6 Hz) as well as the anisotropic effect observed on proline $C^{\delta}H_2$ and $CONH_2$ atoms (see Table 1) suggest for analogue **3** a preferentially staggered conformation of the histidine side chain with its imidazole ring folded on the proline moiety [25].

A further significant spectral feature of the isothiazolidine ring is represented by the ^{1}H and ^{13}C resonances at the C^{γ} position. In agreement with the presence of the SO_2 group, both the ^{1}H and ^{13}C NMR signals appear downfield shifted (δ 3.12, 3.22 and 46.09, respectively) [18,21,29] as compared with the corresponding pGlu resonances in TRH (δ 2.46 and 26.57, respectively; see Table 1).

3. Results and discussion

Our first interest was the investigation of the effects of the pGlu replacement with its sulphonamido counterpart on enzymatic stability towards PP I (EC 3.4.19.3). Metabolic stability was monitored by differential MALDI–TOF MS analysis [30] of TRH analogue **3** and TRH following PP I treatment [31]. While TRH was easily hydrolysed as expected and as

Table 1
NMR data [a] for TRH and TRH analogue **3**

Residue		TRH		**3**	
		δ_H (ppm) [23]	δ_C (ppm) [24]	δ_H (ppm)	δ_C (ppm)
pGlu or (S)-isothiazolidine-1,1-dioxide-3-carboxylic acid					
	C^{α}	4.44	56.83	3.96	55.24
	C^{β}	2.23	30.65	1.98	27.07
		2.46		2.12	
	C^{γ}	2.46	26.57	3.12	46.09
				3.22	
	CO (peptide)		173.56		170.49 [b]
	CO		181.03		
	NH	8.19		7.34	
His	C^{α}	4.98	53.67	4.61	51.55
	C^{β}	3.23	32.63	2.86	30.03
				2.91	
	Im C_2	7.88	136.50	8.08	135.60
	Im C_4	7.30	n.o.	7.62	117.49
	Im C_5		n.o.		133.32
	Im NH	n.o.		11.90	
	CO		171.46		170.01 [b]
	NH	8.58		7.92	
Pro	C^{α}	4.59	61.59	4.16	60.73
	C^{β}	2.23	30.83	2.15	29.97
		2.46	n.o.	2.56	31.83
	C^{γ}	2.23	23.45	1.65	22.89
			25.85	2.02	25.02
	C^{δ}	3.61	47.69	3.36	47.42
		3.89	48.29	3.54	n.o.
	CO		175.54		174.51
	NH_2	7.30		6.94	
		8.51		7.00	

[a] In DMSO at 23 °C.
[b] Assignments may be interchanged.

evidenced by the disappearance after 1 h of its molecular ion (m/z 363), the analogue **3** was found stable to enzymatic hydrolysis, the molecular ion (m/z 399) remaining present and unchanged during the same experiment. This result shows that peptide **3** is significantly stabilized towards hydrolysis by PP I as compared to TRH and may represent a degradation-resistant TRH analogue with prolonged in vivo activity.

In the present pharmacological studies a comparative evaluation of the hormonal effects of TRH and its sulphonamido analogue **3** has been initially considered. TRH is known to induce the secretion of the pituitary lactogenic hormone prolactin (PRL), through hypophyseal lactotropic cell TRH receptor activation. We report here the effects of **3** and TRH on PRL secretion in vitro by using anterior pituitary cell cultures from adult male Wistar rats. It is found that the TRH analogue **3** is able to stimulate PRL secretion in a dose-dependent manner, although with lower potency than TRH (Fig. 2). In particular, the doubling of the control values is obtained with 87.0 nM TRH analogue **3** and 1.2 nM TRH, respectively. Thus, upon modification of the pyroglutamyl moiety, the PRL-releasing activity is retained, albeit to a lesser extent, suggesting that the endocrine TRH receptor binding properties in compound **3** are maintained.

There is a growing evidence that CNS-activating pharmacological effects of TRH are mediated through various neurotransmitters, most prominently catecholamines and acetylcholine [11,32–35]. In a preliminary study we found that TRH is able to inhibit hypothalamic dopamine release from male rat neuronal endings (synaptosomes) in vitro [36]. This suggests a possible TRH involvement either in central mechanisms controlling the anorectic behaviour or in the modulation of PRL release via an indirect pathway; the hypothalamic dopamine is in fact the main putative PRL inhibiting factor (PIF). It seemed then interesting to evaluate here the activity of compound **3** on dopamine release in comparison with TRH, by using rat hypothalamic synaptosomal fractions. It was found that in contrast with native TRH, which is able to inhibit depolarization-induced dopamine release, the TRH analogue **3** has lost this activity (Fig. 3).

4. Experimental

4.1. Peptide synthesis

Column chromatography was carried out on silica gel 60 (230–400 mesh, Merck). TLC was performed on precoated silica gel 60 F_{254} Merck plates, developed with the following solvent systems: (A) $CHCl_3$–MeOH (85:15); (B) $CHCl_3$–MeOH (3:1). Optical rotations were taken at 20 °C with a Schmidt-Haensch Polartronic D polarimeter. ^1H and ^{13}C NMR spectra of the new compounds were determined in DMSO-d_6 solution on a Varian VXR 300 MHz instrument (δ expressed in ppm). Elemental analyses for the new compounds are within the $\pm$ 0.4% of the theoretical values. Compound **1** and its *N*-benzyloxycarbonyl derivative were synthesized as described in Ref. [19].

4.1.1. (*S*,*S*,*S*)-*N*-(2-Benzyloxycarbonyl-isothiazolidine-1,1-dioxide-3-carbonyl)-histidyl-prolinamide (**2**)

To a solution of (*S*)-2-benzyloxycarbonyl-isothiazolidine-1,1-dioxide-3-carboxylic acid (2.8 g, 9.4 mmol) in DMF (40 ml) TEA (1.0 g, 10.3 mmol) and isobutyl chloroformate (1.4 g, 10.3 mmol) were added dropwise alternately at − 10 °C. After 20 min stirring, a solution of 2HCl·H–His–Pro–NH_2 [20] (4.6 g, 14.2 mmol) in DMF (40 ml) and TEA (4.3 g, 42.6 mmol) were added, and the mixture stirred at 0 °C for 3 h and at room temperature (r.t.) for 20 h. The reaction mixture was

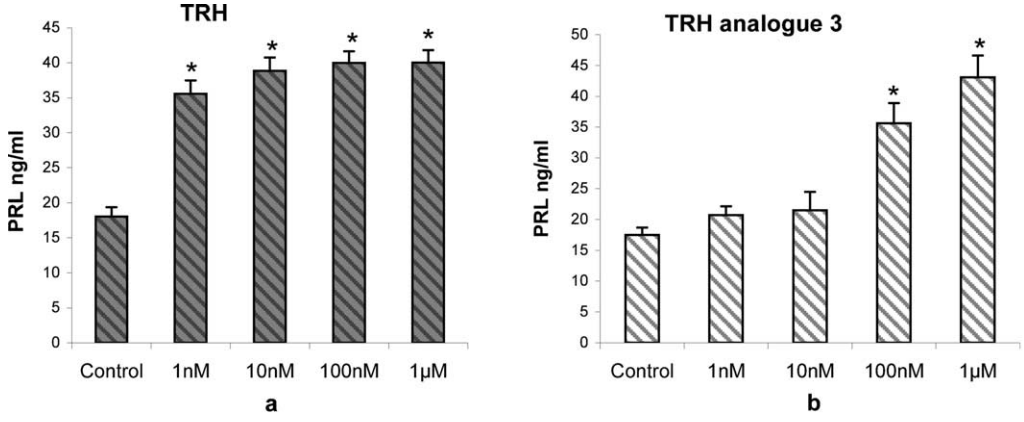


Fig. 2. Effect of TRH (a) and TRH analogue **3** (b) (1 nM–1 μM) on PRL release from anterior pituitary cell cultures. Trypsin dispersed cells were plated into 24-well dishes (300,000 cells/well). After 2–3 days in culture, the cells were washed twice with 1 ml DMEM and release experiments were performed by 1 h incubations with graded concentrations of TRH or TRH analogue **3** in DMEM. Each column represents the mean ± SEM of 12–15 wells; ANOVA $P < 0.0001$, $^*P < 0.001$ vs. control.

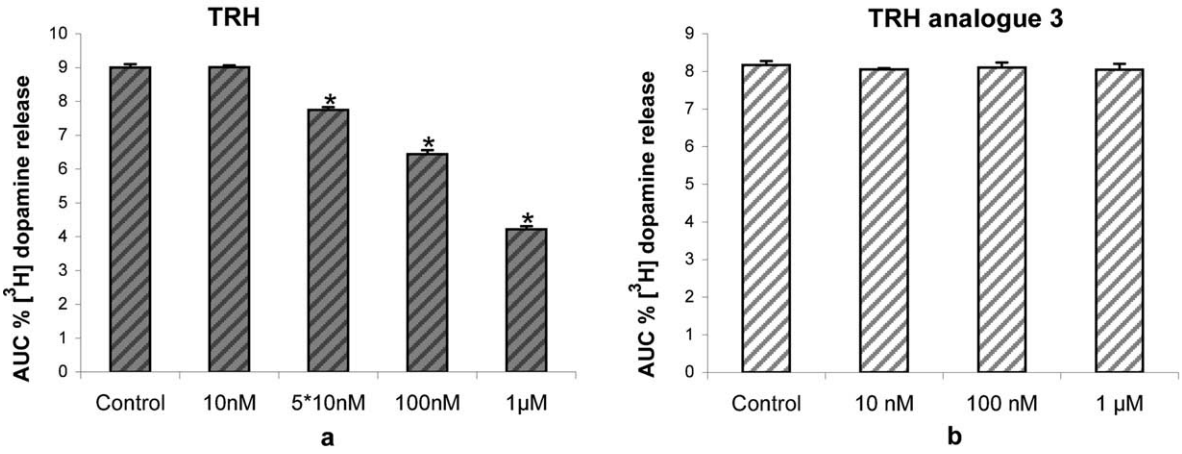


Fig. 3. Areas under the time–response curves (AUCs) relative to the effects of TRH (a) and TRH analogue **3** (b) (10 nM–1 μM) on depolarization-induced dopamine release. After a 30 min equilibration perfusion with buffer alone, a 23 min perfusion with graded concentrations of the peptides was started, where in the final 3 min, K^+ concentrations in the perfusion buffer were elevated to 15 mM (after removal of equimolar concentrations of Na^+). Then, the Krebs–Ringer buffer perfusion was restarted for 9 min to record a return to basal. Controls followed the same protocol, but without peptides in perfusion buffer. Each column represents the mean ± SEM of 3–5 experiments performed in triplicate; ANOVA, $P < 0.0001$; $^*P < 0.001$ vs. controls.

filtered, the solvent removed in vacuo and the residue chromatographed on silica gel using $CHCl_3$–MeOH (85:15) as eluant, to give *N*-protected tripeptide **2** as an oil in 50% yield. *Anal.* ($C_{23}H_{28}N_6O_7S$) C, H, N, S; *Rf* (A) 0.3, *Rf* (B) 0.4; $[\alpha]_D = -55°$ ($c = 1$, MeOH). ^1H NMR: δ 1.7–1.9 (m, 3H, Pro γ-CH_2 and β-CH_B), 2.0 (m, 1H, Pro β-CH_A), 2.25 (m, 1H, SO_2–CH_2–CH_B), 2.55 (m, 1H, SO_2–CH_2–CH_A), 2.7–2.9 (m, 2H, His β-CH_2), 3.1 (m, 1H, Pro δ-CH_B), 3.25–3.65 (m, 3H, SO_2–CH_2 and Pro δ-CH_A), 4.2 (m, 1H, Pro α-CH), 4.55 (m, 1H, His α-CH), 4.7 (m, 1H, SO_2–N(*Z*)–CH), 5.2 (m, 2H, CH_2O), 6.8 (s, 1H, Im C_4), 7.0 (s, 1H, NH), 7.3–7.4 (m, 5H, aromatics), 7.55 (s, 1H, Im C_2), 8.15 (s, 1H, NH), 8.6 (d, $J = 6.46$ Hz, 1H, His NH), 11.8 (s, 1H, Im NH). ^{13}C NMR: δ 22.76 (Pro C^γ), 24.32 (SO_2–CH_2–CH_2), 29.30 (Pro and His C^β), 46.72 (SO_2–CH_2), 47.18 (Pro C^δ), 51.65 (Pro C^α), 57.35 (SO_2–N(*Z*)–CH), 60.03 (His C^α), 67.63 (CH_2O), 117.50 (Im C_4), 127.47, 128.13, and 128.42 (Ph), 133.81 (Im C_5), 134.99 (Ph), 135.39 (Im C_2), 150.05 (OCO), 168.43, 169.71, and 173.94 (3 × CO).

*4.1.2. (S,S,S)-N-(isothiazolidine-1,1-dioxide-3-carbonyl)-histidyl-prolinamide (**3**)*

Peptide **2** (2.2 g, 4.1 mmol) was hydrogenated in MeOH (100 ml) in the presence of 10% Pd on activated charcoal (0.2 g). After 2 h, the catalyst was filtered off and the filtrate evaporated under reduced pressure. The crude product was purified by column chromatography on Sephadex LH-20 using H_2O–MeOH (2:1) as eluant to yield deprotected tripeptide **3** as a foam (1.5 g, 92%). *Anal.* ($C_{15}H_{22}N_6O_5S$) C, H, N, S; *Rf* (B) 0.2, $[\alpha]_D = -41°$ ($c = 1$, MeOH).

4.2. Biological studies

PP I (EC 3.4.19.3) from calf liver was purchased from Sigma. Differential mass spectrum analyses were obtained with a Voyeger-DE (PE Biosystems) instrument. Some aliquots of analogue **3** and TRH (12 μmol) were treated with 7.7 U of PP I in 1 ml of 50 mM potassium phosphate buffer, pH 8.0, containing 5 mM mercaptoethanol and 5 mM EDTA. At different times, 10 μl aliquots were dissolved with an appropriate MALDI matrix in MeCN solution containing 0.1% trifluoroacetic acid.

4.3. Pharmacological studies

4.3.1. Materials and methods

4.3.1.1. Anterior pituitary cell cultures. Cell dispersion was performed as previously described [37], under sterile conditions and the media were supplemented with penicillin (35 μg/ml) and streptomycin (50 μg/ml). The anterior pituitaries were minced with a blade into small fragments and sequentially incubated in Dulbecco's modified eagle's medium (DMEM) at 37 °C in a Dubnoff shaking bath, with 0.5% trypsin (type XII S) for 15 min, 0.02% deoxyribonuclease I for 1 min, 0.1% soybean trypsin inhibitor for 4 min, 2 and 1 mM EDTA (in Ca^{2+}/Mg^{2+}-free Earle's basic salt solution, EBSS) for 4 and 15 min, respectively. The remaining tissue fragments were mechanically dispersed into single cells by gentle suction and extrusion through a narrow tip Pasteur pipette; the cell suspension was then filtered through a 100 μm nylon mesh and centrifuged at

$1000 \times g$ for 10 min through a layer of 3% bovine serum albumin (BSA) in DMEM. Finally, cells were resuspended in DMEM–0.3% BSA, checked for viability by Trypan blue exclusion test, counted by a haemocytometer (ca. 2 million cells/pituitary), plated into 24-well dishes (300,000 cells/well), and incubated with DMEM supplemented with 10% newborn calf serum, at 37 °C in ambient air/CO_2 95/5%. After 2–3 days in culture, the cells were washed twice with 1 ml DMEM and release experiments were performed by 1 h incubations with graded concentrations of TRH or TRH analogue **3** in DMEM (supplemented with 0.1% BSA, 0.006% ascorbic acid and 40 IU/ml aprotinin); release aliquots were stored at −20 °C until assay. Chemicals and cell culture reagents were purchased from Sigma, except DMEM and serum from Gibco.

PRL radioimmunoassay was performed as previously described [37]. PRL was iodinated with $Na^{125}I$ by the chloramine-T method. Rat PRL antibody and standard were provided by NIDDK, National Hormone and Pituitary Program, USA. Hundred microlitres of rat PRL standard or samples were incubated at 5 °C with the PRL antibody at a final dilution of 1:12,500 in assay buffer for 24 h; thereafter, 3000 cpm ^{125}PRL were added to each tube and incubated for 48 h. Finally, *anti*-rabbit goat serum (1:200) and 0.4% polyethyleneglycol were added to separate bound and free fractions. After 24 h tubes were centrifuged for 30 min at $4000 \times g$, supernatants were aspirated, and pellets counted in a γ-counter. The sensitivity of the assay was 50 pg/tube.

4.3.1.2. Hypothalamic synaptosomes. Hypothalamic synaptosomes were prepared according to Ref. [38]. Briefly, male Wistar rats (200–250 g) were sacrificed by decapitation, the hypothalami quickly dissected, homogenized in 0.32 M saccharose and centrifuged, first at $1000 \times g$ for 5 min, and then at $12{,}000 \times g$ for 20 min, to isolate neuronal endings from cell nuclei and glia. Then, the synaptosome suspension was incubated, at 37 °C, under O_2/CO_2 95/5%, pH 7.2–7.4, in Krebs–Ringer buffer (mM/l: 125 NaCl, 3 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 5 NaH_2PO_4, 10 Tris–HCl, 10 glucose, 1, ascorbic acid), supplemented with 0.05 μM fluoxetine, a selective serotonin reuptake inhibitor, with 0.05 μM [^{3}H]dopamine, for 15 min, to make synaptosomes uptake [^{3}H]dopamine, substituting for the endogenous dopamine pool. Then, synaptosomes were layered onto 0.8 μM Millipore filters, placed into 37 °C water-jacketed superfusion chambers (18 different chambers for each experiment), and perfused with the above buffer (0.6 ml/min). After 30 min, to allow stable release (equilibration period), perfusate was collected in 2 min fractions, and after the first 3–4 fractions (basal release) graded concentration of TRH or TRH analogue **3** were added to the perfusion buffer for 10 min (stimulus), followed by 10 min with Krebs buffer alone (return to basal). To evaluate the effects of the peptides on neurotransmitter release during depolarization [3 min perfusion with K^+(15 mM), after removal of equimolar concentrations of Na^+ in perfusion buffer], TRH or TRH analogue **3** were added, after the equilibration period, both 20 min prior (pre-stimulus) and 3 min during K^+(15 mM) perfusion (stimulus). Then, Krebs–Ringer buffer perfusion was restarted for 9 min to record a return to basal. Finally, β-emission from perfusate fractions, corresponding to [^{3}H]dopamine release, was detected by liquid scintillation scanning. All chemicals were purchased from Carlo Erba, Italy, except [7,8-^{3}H]dopamine (1 mCi/ml) from Amersham Pharmacia Biotech, Italy.

4.3.2. Analysis of data

PRL release has been evaluated as group means ± standard error of mean (SEM). Each group represents the mean ± SEM of 12–15 wells.

Dopamine release has been calculated as either the means ± SEM of the percentage of [^{3}H]dopamine recovered in each fraction respect to total (fractions + filter) or the means ± SEM of the area under the time–response curve (AUC); each group represents the mean ± SEM of 3–5 experiments performed in triplicate. Treatment and control group data from experiments run on a single pool of hypothalamic tissue.

Treatment and control group means have been compared by the analysis of variance (ANOVA), followed by the Student–Newman–Keul test (GraphPad Prism 2.00 Software).

5. Conclusions

In summary, we have found that the replacement of the pGlu residue with its sulphonamido counterpart, the (*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid, at the *N*-terminus of TRH stabilizes the scissile Xaa–His amide bond to hydrolysis by the pyroglutamyl peptidases, which represents the main mechanism responsible for the peptide in vivo inactivation. This result is valuable for the design of metabolically stabilized TRH and TRH-like peptide analogues to be considered for clinical trials.

In the pharmacological investigations, analogue **3** was found to retain, albeit with a weaker potency, the hormonal activity of TRH, as measured by the prolactin-releasing effect. When the analogue was tested for its ability to inhibit dopamine release, through activation of hypothalamic TRH receptors, it was found devoid of activity. These differences are in line with emerging data supporting the hypothesis that the endocrine and CNS effects of TRH may be mediated through different TRH receptor subtypes: the high-affinity, pituitary-like binding sites, and the low-affinity binding sites in hypothalamus and cortex [39,40].

Although the pyroglutamic acid moiety is not considered to be involved in the stabilization of the putative bioactive conformation of TRH [23], its cyclic structure and conformation are important in maintaining the right spatial location of the lactam thus stabilizing the hydrogen bond formation in the receptor interaction [41]. By taking into account the conformational analogy between **3** and the native molecule at the level of the His–Pro–NH_2 fragment, the change in the hormonal potency as well as the lack of the CNS activity should be ascribed to alterations in receptor interactions connected with the specific properties of the SO_2NH group. In this context, an unfavourable electrostatic potential of sulphonamides [42] and/or the presence of the more sterically demanding tetrahedral structure of the SO_2 group as compared with the planar amide carbonyl should play an important role.

Acknowledgements

Financial support from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, 60%) (Italy) is gratefully acknowledged. The authors wish to thank Professor G. Lucente for his suggestions and helpful discussions.

References

[1] P.G. Board, K.A. Moore, J.E. Smith, Purification and properties of gamma-glutamylcyclotransferase from human erythrocytes, Biochem. J. 173 (1978) 427–431.
[2] W.H. Fischer, J. Spiess, Identification of a mammalian glutaminyl cyclase converting glutaminyl into pyroglutamyl peptides, Proc. Natl. Acad. Sci. USA 84 (1987) 3628–3632.
[3] W.H. Busby Jr., G.E. Quackenbush, J. Humm, W.W. Youngblood, J.S. Kizer, An enzyme(s) that converts glutaminyl-peptides into pyroglutamyl-peptides. Presence in pituitary, brain, adrenal medulla, and lymphocytes, J. Biol. Chem. 262 (1987) 8532–8536.
[4] L. Prokai, X. Ouyang, K. Prokai-Tatrai, J.W. Simpkins, N. Bodor, Synthesis and behavioral evaluation of a chemical brain-targeting system for a thyrotropin-releasing hormone analogue, Eur. J. Med. Chem. 33 (1998) 879–886.
[5] P.M. Cummins, B. O'Connor, Pyroglutamyl peptidase: an overview of the three known enzymatic forms, Biochim. Biophys. Acta 1429 (1998) 1–17.
[6] L. Flohé, K. Bauer, E. Friderichs, W.A. Günzler, H.H. Hennies, S. Herrling, F. Lagler, F. Ötting, E. Schwertner, Biological effects of degradation-stabilized TRH analogues, in: E.C. Griffiths, G.W. Bennett (Eds.), Thyrotropin Releasing Hormone, Raven Press, New York, 1983, pp. 327–340.
[7] R.F. Nutt, F.W. Holly, C. Homnick, R. Hirschmann, D.F. Veber, B.H. Arison, Synthesis of thyrotropin-releasing hormone analogues with selective central nervous system effects, J. Med. Chem. 24 (1981) 692–698.
[8] G. Metcalf, Regulatory peptides as a source of new drugs—the clinical prospects for analogues of TRH which are resistant to metabolic degradation, Brain Res. 257 (1982) 389–408.
[9] C. Mapelli, L.F. Elrod, F.L. Switzer, C.H. Stammer, Conformational properties of 2,3-methanopyroglutamic acid in peptides: NMR and X-ray diffraction studies, Biopolymers 28 (1989) 123–128.
[10] B. Koksch, D. Ullmann, H.-D. Jakubke, N. Sewald, K. Burger, Structure and biological activity of trifluoromethyl substituted GnRH and TRH analogues, Peptides (1994) 323–324.
[11] I. Fukuchi, T. Asahi, K. Kawashima, Y. Kawashima, M. Yamamura, Y. Matsuoka, K. Kinoshita, Effects of taltirelin hydrate (TA-0910), a novel thyrotropin-releasing hormone analog, on in vivo dopamine release and turnover in rat brain, Arzneim.-Forsch./Drug Res. 48 (1998) 353–359.
[12] A. Calcagni, M. Kajtar-Peredy, G. Lucente, G. Luisi, F. Pinnen, L. Radics, D. Rossi, Nine-membered cyclodepsitripeptides containing the retroisomeric sequence of ergot peptides, Int. J. Pept. Protein Res. 42 (1993) 84–92.
[13] A. Calcagni, S. Duprè, G. Lucente, G. Luisi, F. Pinnen, D. Rossi, A. Spirito, Synthesis and activity of the glutathione analogue γ-(L-(γ-oxaglutamyl)-L-cysteinyl-glycine, Arch. Pharm. Pharm. Med. Chem. 329 (1996) 498–502.
[14] A. Calcagni, G. Lucente, G. Luisi, F. Pinnen, D. Rossi, E. Gavuzzo, Approaches to pseudopeptidic ergopeptines. Part 3. Consequences of the incorporation of an α-azaphenylalanine residue into the ergotamine oxa-cyclolic system, J. Chem. Soc., Perkin Trans 1 (1997) 2223–2227.
[15] A. Calcagni, G. Lucente, G. Luisi, F. Pinnen, D. Rossi, Novel glutathione analogues containing the dithiol and disulfide form of the Cys–Cys dyad, Amino acids 17 (1999) 257–265.
[16] G. Luisi, A. Calcagni, F. Pinnen, Ψ(SO_2–NH) Transition state isosteres of peptides. Synthesis of the glutathione disulfide analogue [γ-Glu–Ψ(SO_2–NH)–Cys–Gly]$_2$, Tetrahedron Lett. 34 (1993) 2391–2392.
[17] A. Calcagni, D. Rossi, M. Paglialunga Paradisi, G. Lucente, G. Luisi, E. Gavuzzo, F. Mazza, G. Pochetti, M. Paci, Peptides containing the sulfonamide junction. Synthesis, structure and conformation of *Z*-Tau–Pro–Phe–NHiPr, Biopolymers 41 (1997) 555–567.
[18] A. Calcagni, P.G. Ciattini, A. Di Stefano, S. Duprè, G. Luisi, F. Pinnen, D. Rossi, A. Spirito, ψ(SO_2NH) Transition state isosteres of peptides. Synthesis and bioactivity of sulfonamido pseudopeptides related to carnosine, Farmaco 54 (1999) 673–677.
[19] G. Luisi, F. Pinnen, Synthesis and properties of (*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid, a new γ-sultam analogue of pyroglutamic acid, Arch. Pharm. 326 (1993) 139–141.
[20] M. Bienert, Gy. Köller, H. Niedrich, Synthese von Thyreoliberin (TRF), Pharmazie 32 (1977) 397–398.
[21] A. Calcagni, E. Gavuzzo, G. Lucente, F. Mazza, F. Pinnen, G. Pochetti, D. Rossi, Structure and conformation of peptides containing the sulfonamide junction. II. Synthesis and conformation of cyclo[–MeTau–Phe–D-Pro–], Int. J. Peptide Protein Res. 34 (1989) 471–479.
[22] W.F. Erman, H.C. Kretschmar, Syntheses and facile cleavage of five-membered ring sultams, J. Org. Chem. 26 (1961) 4841–4850.
[23] M. Montagut, B. Lemanceau, A.-M. Bellocq, Conformational analysis of thyrotropin releasing factor by proton magnetic resonance spectroscopy, Biopolymers 13 (1974) 2615–2629.
[24] R. Deslauriers, C. Garrigou-Lagrange, A.-M. Bellocq, I.C.P. Smith, Carbon-13 nuclear magnetic resonance studies on thyrotropin-releasing factor and related peptides, FEBS Lett. 31 (1973) 59–66.
[25] B. Donzel, J. Rivier, M. Goodman, Conformational studies on the hypothalamic thyrotropin releasing factor and related compounds by ^{1}H nuclear magnetic resonance spectroscopy, Biopolymers 13 (1974) 2631–2647.
[26] H. Kessler, Conformation and biological activity of cyclic peptides, Angew. Chem. Int. Ed. Engl. 21 (1982) 512–523.

[27] J. Feeney, G.R. Bedford, P.L. Wessels, ^{1}H Nuclear magnetic resonance studies of thyrotropin releasing factor (TRF), FEBS Lett. 42 (1974) 347–351.
[28] S. Fermandjian, P. Pradelles, P. Fromageot, Proton NMR studies on thyrotropin releasing factor, FEBS Lett. 28 (1972) 156–160.
[29] A. Calcagni, E. Gavuzzo, G. Lucente, F. Mazza, F. Pinnen, G. Pochetti, D. Rossi, Structure and conformation of peptides containing the sulphonamide junction. III. Synthesis, crystal and molecular structure of a taurine containing peptidic oxa-cyclol, Int. J. Peptide Protein Res. 37 (1991) 167–173.
[30] B.T. Chait, S.B.H. Kent, Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins, Science 257 (1992) 1885–1894.
[31] A. Szewczuk, M. Mulczyk, Pyrrolidonyl peptidase in bacteria. The enzyme from *Bacillus subtilis*, Eur. J. Biochem. 8 (1969) 63–67.
[32] H.H. Keller, G. Bartholini, A. Pletscher, Enhancement of cerebral noradrenaline turnover by thyrotropin-releasing hormone, Nature 248 (1974) 528–529.
[33] G.G. Yarbrough, TRH potentiates excitatory actions of acetylcholine on cerebral cortical neurons, Nature 263 (1976) 523–524.
[34] A. Horita, M.A. Carino, H. Lai, Pharmacology of thyrotropin releasing hormone, Annu. Rev. Pharmacol. Toxicol. 26 (1986) 311–332.
[35] A. Horita, An update on the CNS actions of TRH and its analogs, Life Sci. 62 (1998) 1443–1448.
[36] L. Brunetti, G. Orlando, B. Michelotto, L. Recinella, M. Vacca, Cocaine- and amphetamine-regulated transcript peptide-(55-102) and thyrotropin releasing hormone inhibit hypothalamic dopamine release, Eur. J. Pharmacol. 409 (2000) 103–107.
[37] L. Brunetti, P. Preziosi, E. Ragazzoni, M. Vacca, Effects of vindesine on hypothalamic–pituitary–adrenal axis, Toxicol. Lett. 75 (1995) 69–73.
[38] L. Brunetti, B. Michelotto, G. Orlando, M. Vacca, Leptin inhibits norepinephrine and dopamine release from rat hypothalamic neuronal endings, Eur. J. Pharmacol. 372 (1999) 237–240.
[39] H. Asai, K. Kinoshita, M. Yamamura, Y. Matsuoka, Diversity of thyrotropin-releasing hormone receptors in the pituitary and discrete brain regions of rats, Jpn. J. Pharmacol. 79 (1999) 313–317.
[40] S. Vonhof, G.Z. Feuerstein, L.A. Cohen, V.M. Labroo, Norvaline^{2}-TRH: binding to TRH receptors in rat brain homogenates, Eur. J. Pharmacol. 180 (1990) 1–12.
[41] H.J. Goren, L.G. Bauce, W. Vale, Forces and structural limitations of binding of thyrotrophin-releasing factor to the thyrotrophin-releasing receptor: the pyroglutamic acid moiety, Mol. Pharm. 13 (1977) 606–614.
[42] J.L. Radkiewicz, M.A. McAllister, E. Goldstein, K.N. Houk, A theoretical investigation of phosphonamidates and sulfonamides as protease transition state isosteres, J. Org. Chem. 63 (1998) 1419–1428.