ELSEVIER

Journal of Fluorine Chemistry 80 (1996) 53–57

JOURNAL OF FLUORINE CHEMISTRY

Synthesis, structure and biological activity of α-trifluoromethyl-substituted thyreotropin releasing hormone [1]

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Accepted 1 January 1996

Abstract

The incorporation of α-trifluoromethyl pyroglutamic acid into thyreotropin releasing hormone TRH, results in the complete stability of the *p*Glu–His bond against proteolytic degradation by pyroglutamyl aminopeptidase II. The influence of the trifluoromethyl group on structure and receptor affinity has been studied.

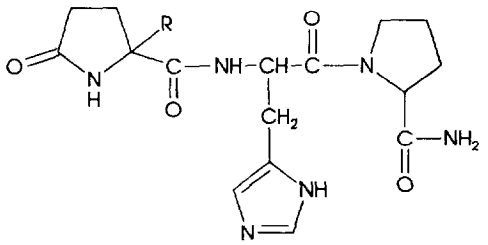
Keywords: α-Trifluoromethyl-substituted amino acids; TRH; Proteolytic stability; NMR spectroscopy; IR spectroscopy

1. Introduction

The incorporation of α,α-disubstituted amino acids into peptides is an efficient strategy for retarding proteolytic degradation and stabilizing secondary structures [1].

Due to the unique electronical properties of fluoro substituents, the α-trifluoromethyl-substituted amino acids (α-TFM amino acids) are a special class of α,α-disubstituted amino acids and are therefore interesting building blocks for peptide synthesis [2]. A trifluoromethyl group in an α-position of an amino acid exerts considerable polarization effects on neighbouring substituents. This structural alteration influences the hydrolytic stability of peptides containing TFM amino acids, resulting in retarded degradation by peptidases [3], and consequently, in prolonged intrinsic activity. The steric bulk of a trifluoromethyl group is still a controversial issue; it should be close to that of an isopropyl group [4]. Undoubtedly, it exerts severe conformational restrictions on these peptides. Furthermore, due to the high electron density, the trifluoromethyl group is capable of participating in hydrogen bonding. We have started incorporating α-TFM amino acids into relevant functional positions in biological active peptides to investigate the steric, electronic and hydrophobic effects of a trifluoromethyl group on proteolytic stability and biological activity.

A peptide as simple structurally as TRH (thyreotropin releasing hormone: *p*Glu–His–Pro–NH_2) (Scheme 1), with a high biological activity as well as pronounced specificity, represents a model of choice for the synthesis of analogues to study structure–activity relationships. TRH is the central stimulator of TSH (thyroid stimulating hormone) secretion by anterior pituitary cells, and is equally capable of stimulating the release of prolactin and growth hormone [5]. TRH is regulated by peripheral and pituitary hormone levels, and is used for the treatment of various neurologic and neuropsychiatric disorders [6]. The degradation of TRH in vivo is initiated by pyroglutamyl aminopeptidase II which selectively cleaves the *p*Glu–His bond [7]. Of the numerous analogues of TRH which have been synthesized so far, only four exhibit higher activity than TRH itself in releasing TSH. [2-(*N*-MethylHis)]TRH and the corresponding *N*-amylamide have eight-times and approximately 10-times the potency of TRH, respectively [8].



Scheme 1. Structure of TRH; R = H: native peptide, R = TFM: [(α-TFM)*p*Glu[1]]-TRH.

Substitution of *p*Glu by (α-TFM)*p*Glu at position 1 of TRH is assumed to protect this hormone against hydrolysis by pyroglutamyl aminopeptidase II. The introduction of the

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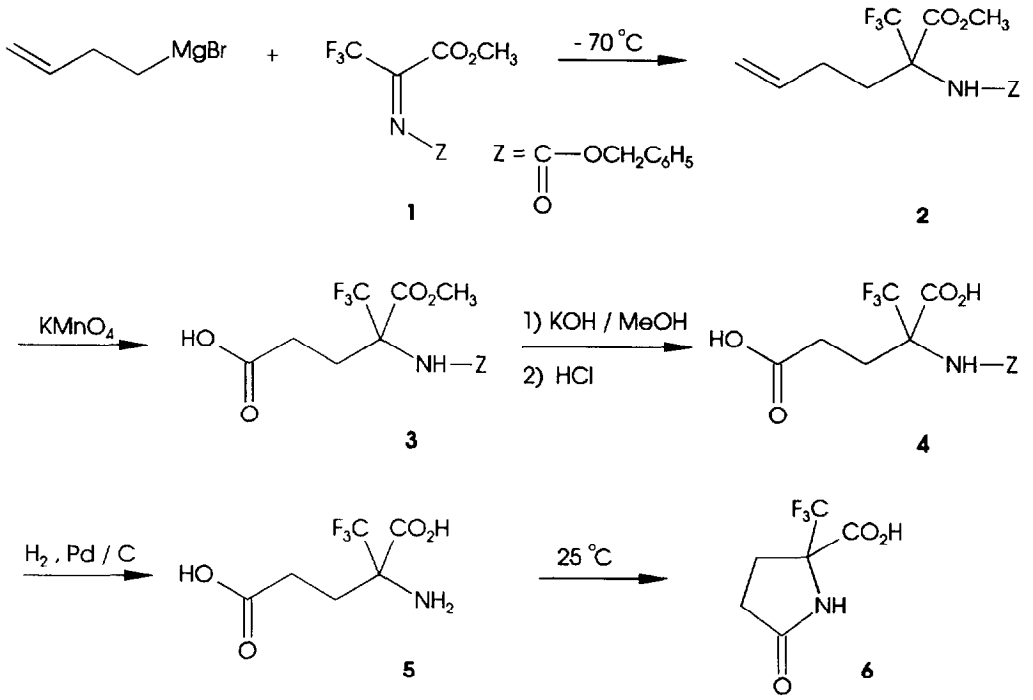
[1] Dedicated to Prof. Dr. Ivar Ugi on the occasion of his 65th birthday.

PII S0022-1139(96)03461-6



Scheme 2. Synthesis of (α-TFM)*p*Glu.

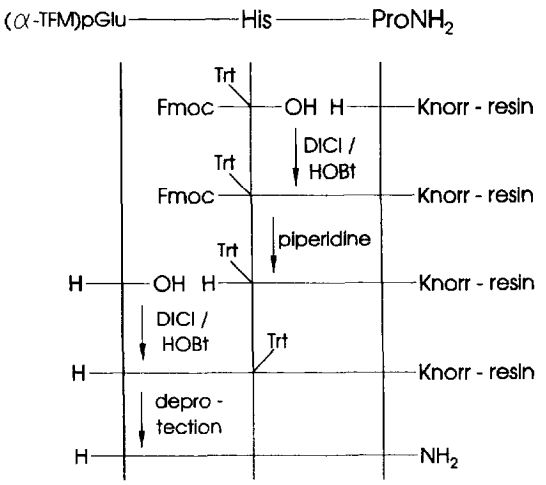
strong electron-withdrawing trifluoromethyl group should profoundly influence the charge distribution and the three-dimensional conformation, and hence the interaction between substrate and receptor profoundly.

2. Results and discussion

α-TFM-substituted *p*Glu was obtained from methyl [2-(*N*-benzyloxycarbonylimino)]-3,3,3-trifluoropropanoate (**1**) via cyclization of the unstable α-TFM glutamic acid (**5**→**6**) at room temperature [9] (Scheme 2). Compound **6** was introduced into the TRH sequence by solid-phase peptide synthesis using the Knorr resin (Scheme 3). To completely couple (α-TFM)*p*Glu on the peptide resin, a 4–5-fold excess of the corresponding amino acid and of the coupling reagents, as well as a prolonged reaction time of 8 h, was necessary. The resulting analogue was purified by reversed-phase high-performance liquid chromatography (RPHPLC). Characterization was achieved by FAB MS and NMR spectroscopy.

The metabolic stability of [(α-TFM)*p*Glu^1]-TRH against cleavage by membrane-bound TRH degrading ectoenzyme from Wistar rat anterior pituitary cells was determined in an assay, testing the inhibition rate of *p*Glu[^{3}H]TRH degradation (Fig. 1) [10]. As expected, the degradation of *p*Glu[^{3}H]TRH is not effectively inhibited by [(α-TFM)*p*Glu^1]-TRH. Thus, trifluoromethyl substitution at position 1 results in complete resistance to proteolysis.

The receptor binding affinity of (α-TFM)*p*Glu-substituted TRH was determined as its potency in decreasing the [^{3}H]CH_3–TRH receptor binding. As demonstrated in Fig. 2, the receptor affinity of the trifluoromethyl-substituted ana-



Scheme 3. Synthesis of [(α-TFM)*p*Glu^1]-TRH.

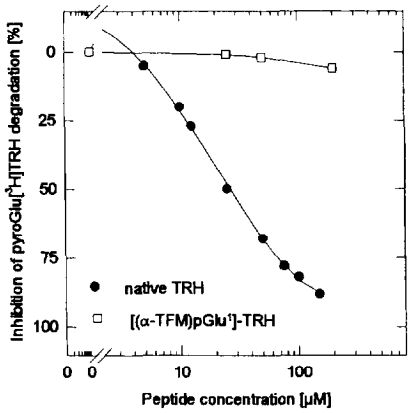


Fig. 1. Determination of the degradation stability of [(α-TFM)*p*Glu^1]-TRH by *p*Glu[^{3}H]TRH degradation assay.

Table 1
^{1}H NMR shift values of native TRH and the diastereomers of [(α-TFM)*p*Glu^1]–TRH

Residue	Group	^{1}H NMR shift value, δ (ppm); J(Hz)	
		Native TRH	[(α-TFM)*p*Glu^1]–TRH
*p*Glp	NH	7.77 (s, 1H)	8.88/8.89 (s/s, 1H/1H)
	α	3.99 (m, 1H)	–
	β	1.70 (m, 1H); 2.14 (m, 1H)	1.78/2.35 (m/m, 1H/1H)
	γ	2.00 (m, 2H)	2.25 (m, 2H)
His	NH	8.30 (d, 1H, J=8.0 Hz)	8.41/8.46 (d/d, J=7.93/8.24 Hz)
	α	4.78–4.84 (m, 1H)	4.80–4.86 (m, 1H); 4.88–4.94 (m, 1H); 2.94–3.15 (m, 2H)
	β	2.94 (dd_{AB}, J=15.4 Hz, J=7.3 Hz); 3.07 (dd_{AB}, J=15.4 Hz, J=5.1 Hz)	
	C_2H	8.93 (s, 1H)	8.96–8.98 (m, 1H)
	C_4H	7.40 (s, 1H)	7.35–7.37 (m, 1H)
Pro	NH	–	–
	α	4.23 (m, 1H)	4.22 (m, 1H)
	β	1.75 (m, 1H); 2.05 (m, 1H)	1.81 (m, 1H); 2.04–2.17 (m, 1H)
	γ	1.82 (m, 1H); 1.90 (m, 1H)	1.84–1.89 (m, 2H)
	δ	3.54 (m, 1H); 3.57 (m, 1H)	3.53–3.57 (m, 2H)
	NH_2	7.07 (s, 1H); 7.51 (s, 1H)	7.07/7.55 (s/s, 1H/1H)

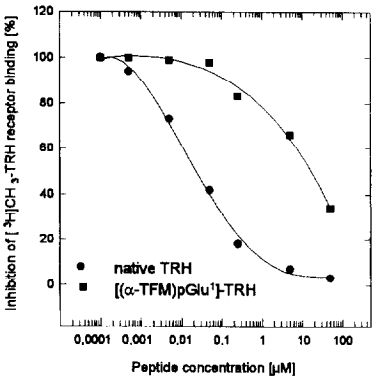


Fig. 2. Inhibition of receptor binding of [^{3}H]CH_3–TRH to Wistar rat anterior pituitary cells by native TRH and [(α-TFM)*p*Glu^1]–TRH.

logue is two to three orders of magnitude less than that of the native compound. Comparison of the NMR shift values of the native sequence and the α-TFM-substituted analogue implies a significant change in the chemical environment in the *p*Glu–His region (Tables 1 and 2). A downfield shift is observed, particularly for the NH groups of *p*Glu and His and also for the C_α atoms. The introduction of the trifluoromethyl group and the stereochemistry at C_α of (α-TFM)*p*Glu seem to have an influence on the ability of the *p*Glu residue to participate in hydrogen bonding. The polarization effect of the α-TFM group obviously decreases the capacity of the *p*Glu residue to form a hydrogen bond between the *p*Glu carboxy function and the amino function of the Pro moiety (Fig. 3). This fact together with the steric constraints of the substituent might prevent a stable hairpin turn required for an optimal interaction with the receptor.

3. Experimental details

Melting points (not corrected) were determined using a Tottoli apparatus (Büchi SMP-20); elemental microanalyses

Table 2
^{13}C NMR shift values of native TRH and the diastereomers of [(α-TFM)*p*Glu^1]–TRH

Residue	Group	^{13}C NMR shift value, δ (ppm); J(Hz)	
		Native TRH	[(α-TFM)*p*Glu^1]–TRH
*p*Glp	TFM		125.05 (q, J=284 Hz)
	α	55.50	66.8 (q, J=28 Hz)
	β	25.14	25.96/26.10
	γ	29.19	28.78
	CO	172.47	167.78/167.95
	CO_{Lactam}	177.56	177.22/177.29
His	α	49.73	50.14/50.58
	β	26.45	26.37
	C_2	133.96	134.07/134.14
	C_4	117.56	117.72/117.82
	C_5	128.97	128.07/128.91
	CO	168.28	166.54/166.72
Pro	α	59.74	59.90/59.96
	β	29.63	29.65/29.69
	γ	24.55	24.57/24.63
	δ	47.03	46.03/47.10
	CO	173.97	173.94

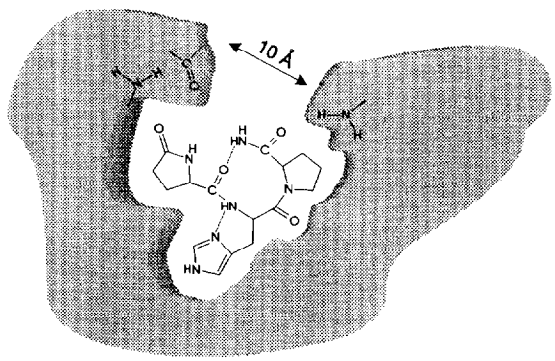


Fig. 3. Model of the interaction between the TRH molecule and the receptor [11].

were carried out with a Heraeus CHN-Elemental Analyzer. IR spectra were recorded using Perkin–Elmer 157 G or 257 spectrophotometers; ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded with a Bruker AM 360 spectrometer at 360, 90 and 339 MHz, respectively. ^{19}F NMR spectra were obtained using JEOL FX 90 Q (84 MHz) and Bruker AC 250 (235 MHz) spectrometers. As reference standard, TMS was used for ^{1}H and ^{13}C spectra (internal) and trifluoroacetic acid for ^{19}F NMR spectra (external). Mass spectra were recorded from electron ionization (EI, 70 eV) with a Varian MAT CH5 instrument.

The solid-phase synthesis was carried out on a semi-automatic batch peptide synthesizer SP650 (Labortec AG, Switzerland) using Knorr resin (Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)benzhydrylamine linked to the aminomethyl resin; 0.55 mmol g^{-1}). Fmoc amino acids were obtained from Bachem (Switzerland), Neosystem (France) or Propeptide (France). The side-chain functional group of His was protected using the trityl group.

3.1. Syntheses

3.1.1. Synthesis of N-(2-trifluoromethyl)pyroglutamic acid (6)

3.1.1.1. Preparation of methyl [2-(N-benzyloxycarbonylamino)]-2-trifluoromethyl-5-hexenoate (2)

Under a nitrogen atmosphere, 2.89 g (10 mmol) of methyl [2-(*N*-benzyloxycarbonylimino)]-3,3,3-trifluoropropanoate (**1**) [12] in 50 ml of absolute diethyl ether were added to a stirred solution consisting of 1.35 g (10 mmol) of 4-bromo-1-butene and 0.24 g (10 mmol) of magnesium in 150 ml of absolute diethyl ether at −70 °C. After warming up to room temperature, the reaction mixture was poured onto 100 ml of ice water, acidified with hydrochloric acid (1 M) and extracted twice with diethyl ether. The combined organic layer was washed with water, dried (Na_2SO_4) and evaporated. The product was purified by distillation in vacuo. Yield: 2.48 g (72%) of **2** as a colourless oil (b.p. 160 °C/0.01 mbar).

IR (film) $\nu(cm^{-1})$: 3420–3340; 1790; 1510. ^{1}H NMR ($CDCl_3$) δ: 1.74–2.47 (m, 4H); 3.84 (s, 3H); 4.91–5.04 (m, 2H); 5.11–5.13 (m, 2H); 5.70 (m, 1H); 5.88 (s, 1H); 7.28–7.38 (m, 5H) ppm. ^{13}C NMR ($CDCl_3$) δ: 27.59, 27.61, 53.90, 65.77 (q, $J=29$ Hz); 67.06, 116.01, 123.90 (q, $J=287$ Hz); 128.12; 128.28; 128.52; 135.87; 153.63; 167.13 ppm. ^{19}F NMR ($CDCl_3$) δ: 4.26 (s, 3F) ppm. Analysis: Calc. for $C_{16}H_{18}F_3NO_4$: C, 55.65; H, 5.25; N, 4.06%; M^+, 345. Found: C, 55.74; H, 5.25; N, 4.06%; M^+, 345.

3.1.1.2. Preparation of 1-Methyl [2-(N-benzyloxycarbonylamino)]-2-trifluoromethyl glutamate (3)

To a stirred solution consisting of 1.72 g (5 mmol) of methyl [2-(*N*-benzyloxycarbonylamino)]-2-trifluoromethyl-5-hexenoate (**2**) in 10 ml of 3 N sulphuric acid and 70 ml of acetone at 0 °C, 5 g (30 mmol) of potassium permanganate in 150 ml of water were slowly added. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 24 h. Sulphuric acid (15 ml) and $NaHSO_3$ (in small portions) were added to the reaction mixture until the solution became colourless. The reaction mixture was evaporated, the residue extracted with diethyl ether and dried (Na_2SO_4). The remaining product was dissolved in sat. $NaHCO_3$, the precipitate separated and the solution carefully acidified with hydrochloric acid. The mixture was extracted with diethyl ether, the combined organic layer dried (Na_2SO_4) and evaporated to dryness. Yield: 1.37 g (76%) of **3** as a colourless oil.

IR (film) $\nu(cm^{-1})$: 3400; 1740; 1720; 1510. ^{1}H NMR ($CDCl_3$) δ: 2.10–2.58 (m, 4H); 3.84 (s, 3H); 5.11 (s, 2H); 5.92 (s, 1H); 7.32–7.39 (m, 5H) ppm. ^{13}C NMR ($CDCl_3$) δ: 24.22, 28.14, 54.12, 65.23 (q, $J=29$ Hz); 67.47, 123.30 (q, $J=288$ Hz); 128.22; 128.45; 128.63; 135.62; 153.96; 166.61; 177.34 ppm. ^{19}F NMR ($CDCl_3$) δ: 4.20 (s, 3F) ppm. Analysis: Calc. for $C_{15}H_{16}F_3NO_6$: C, 49.62; H, 4.40; N, 3.85%; M^+, 363. Found: C, 49.29; H, 4.35; N, 3.82%; M^+, 363.

3.1.1.3. Preparation of 2-(N-benzyloxycarbonylamino)-2-trifluoromethyl glutamic acid (4)

A solution consisting of 1.81 g (5 mmol) of 1-methyl [2-(*N*-benzyloxycarbonylamino)]-2-trifluoromethyl glutamate (**3**) in 50 ml of sodium hydroxide (5%) was stirred at room temperature for 24 h. The reaction mixture was extracted twice with diethyl ether. The aqueous phase was acidified with precooled hydrochloric acid (1 M) and extracted with diethyl ether. The combined organic layer was dried (Na_2SO_4) and evaporated to dryness in vacuo. The remaining product was purified by recrystallization from chloroform/hexanes. Yield: 1.39 g (80%) of **4**, m.p. 152–154 °C.

IR (film) $\nu(cm^{-1})$: 3390; 3160; 1750; 1725; 1700; 1515. ^{1}H NMR ($CDCl_3$) δ: 2.55–2.70 (m, 4H); 5.12 (s, 2H); 5.68 (s, 1H); 7.30–7.37 (m, 5H) ppm. ^{13}C NMR ($CDCl_3$) δ: 26.61, 28.29, 64.91 (q, $J=28$ Hz); 67.04, 125.41 (q, $J=287$ Hz); 128.47; 128.60; 129.02; 137.33; 154.97; 167.06; 173.45 ppm. ^{19}F NMR ($CDCl_3$) δ: 4.15 (s, 3F) ppm. Analysis: Calc. for $C_{14}H_{14}F_3NO_6$; C, 48.15; H, 4.04; N, 4.01%; M^+, 349. Found: C, 47.48; H, 4.03; N, 4.01%; M^+, 349.

3.1.1.4. Preparation of N-(2-trifluoromethyl)pyroglutamic acid (6)

A suspension consisting of 1.74 g (5 mmol) of 2-(*N*-benzoxycarbonylamino)-2-trifluoromethyl glutamic acid (**4**) and 0.5 g of palladium on charcoal in 60 ml of methanol/water (2:1) was stirred at room temperature under a hydrogen atmosphere for 12 h. The catalyst was separated by filtration, the solvent evaporated and the remaining product thoroughly washed with diethyl ether. The crude product was purified by recrystallization from methanol. Yield: 0.94 g (96%) of **6**, m.p. 188–190 °C.

IR (film) $\nu(cm^{-1})$: 3220; 1740; 1625. ^1H NMR (methanol-d_4) δ: 2.43–2.61 (m, 4H) ppm. ^{13}C NMR (methanol-d_4) δ: 26.70, 30.12, 68.62 (q, $J=29$ Hz); 125.91 (q, $J=284$ Hz); 169.67; 180.03 ppm. ^{19}F NMR (methanol-d_4) δ: -0.27 (s, 3F) ppm. Analysis: Calc. for $C_6H_6F_3NO_3$: C, 36.56; H, 3.07; N, 7.11%; M^+, 197. Found: C, 36.51; H, 3.47; N, 7.10%; M^+, 197.

3.1.2. Solid-phase synthesis of [(α-TFM)pGlu¹]–TRH

Coupling reactions were performed with a threefold excess of Fmoc-protected amino acids and DICI/HOBT for 1.5 h. The resulting peptide was cleaved from the resin using a mixture consisting of 95% TFA, 2.5% H_2O and 2.5% ethanedithiol. The diastereomers obtained were precipitated with absolute diethyl ether, washed extensively with diethyl ether, dried (Na_2SO_4) and evaporated to dryness. The crude product was desalted by size exclusion chromotography (SEC; Sephadex LH20; methanol/H_2O, 1:9) and purified by RPHPLC using a gradient system (eluent: 100% A to 65% B in 30 min; [A] = 95% H_2O: 5% CH_3CN: 0.1% TFA; [B] = 5% H_2O: 95% CH_3CN: 0.1% TFA; column; Vydac 218TP108, $\lambda = 280$ nm; flow = 4 ml min^{-1}). After lyophilization and drying over P_2O_5, the products could be isolated as trifluoroacetates. Yield: 21%. We have failed so far to separate the diastereomers. The subsequent tests had to be carried out with the diastereomeric mixture.

^{19}F NMR (DMSO-d_6) δ: 2.8 (s, 3F)/3.0 (s, 3F) ppm. For ^1H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) spectroscopic data, see Tables 1 and 2.

3.2. Biological tests

3.2.1. Proteolytic stability

The protease stability of the trifluoromethyl-substituted analogue was determined in an assay testing the inhibition rate of pGlu[^{3}H]TRH degradation by membrane bound TRH-degrading ectoenzyme from Wistar rat anterior pituitary cells. Pituitary cells in DMEM culture medium supplemented with BSA were incubated with pGlu[^{3}H]TRH and native or trifluoromethyl-substituted TRH at the indicated concentrations. For determination of [^{3}H]TRH degradation by the ectoenzyme, aliquots of the mixture were withdrawn and subjected to ion-exchange chromatography on cellulose phosphate. TRH remained strongly absorbed on this material while the acidic radiolabelled split product pGlu[^{3}H] was quantitatively removed after washing. The radioactivity was measured in the column eluate.

3.2.2. Receptor affinity

The receptor binding affinities of native and trifluoromethyl-substituted TRH were determined as their potency in decreasing the [^{3}H]CH_3–TRH receptor binding. A higher concentration of [(α-TFM)pGlu¹]–TRH was necessary to obtain a comparable effect.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft. We thank Prof. Karl Bauer (Max-Planck-Institute of Experimental Endocrinology, Hannover, Germany) for testing the proteolytic stability and receptor affinity of the TRH analogue. We also thank Mrs. Doris Haines for skillful technical assistance.

References

[1] C. Toniolo, and E. Benedetti, *Macromolecules, 24* (1991) 4004.
[2] N. Sewald and K. Burger, in V.P. Kukhar and V.A. Soloshonok (eds.), *Fluorine-containing Amino Acids, Synthesis and Properties*, John Wiley & Sons, Chichester, UK, 1995, pp. 139–220, and literature cited therein.
[3] K. Burger, K. Mütze, W. Hollweck, B. Koksch, P. Kuhl, H.-D. Jakubke, J. Riede and A. Schier, *J. Prakt. Chem., 335* (1993) 321.
[4] T. Nagai, G. Nishioka, M. Koyama, A. Ando, T. Miki and I. Kumadaki, *J. Fluorine Chem., 57* (1992) 229.
[5] A. Ladram, M. Bulant, A. Delfour, J.J. Montagne, H. Vaudry and P. Nicolas, *Biochimie, 76* (1994) 320.
[6] G. Metcalf and I.M.D. Jackson, *Ann. N.Y. Acad. Sci., 553* (1989) 1.
[7] K. Bauer, P. Nowak and H. Kleinkauf, *Eur. J. Biochem., 118* (1981) 173.
[8] W. König; *Peptide and Protein Hormones: Structure, Regulation, Activity*, VCH, Weinheim/New York/Basel/Cambridge, 1993.
[9] K. Burger and K. Gaa, *Chem.-Ztg., 114* (1990) 101.
[10] K. Bauer, P. Carmelit, M. Schulz, M. Baes and C. Denef, *Endocrinology, 127* (1990) 1224.
[11] G. Grant, N. Ling, J. Rivier and W. Vale, The hormones of the hypothalamus, *Biochemistry, 11* (1972) 3070.
[12] K. Burger, E. Höß and K. Gaa, *Chem.-Ztg., 113* (1989) 243.