[Chem. Pharm. Bull. 28(6)1667—1672(1980)]

Synthesis and Pharmacology of TRH Analogs to separate Central Nervous Action from Endocrine Activity[1)]

Naohisa Fukuda, Osamu Nishimura, Mutsushi Shikata, Chitoshi Hatanaka, Masaomi Miyamoto, Yoshiaki Saji, Ryo Nakayama, Masahiko Fujino and Yuji Nagawa[2)]

Central Research Division, Takeda Chemical Industries Ltd.[2)]

(Received September 12, 1979)

Various TRH analogs, γ-butyrolactone-γ-carbonyl-His-Pro-NHR, γ-butyrolactone-γ-carbonyl-N^{3-im}-methyl-His-Pro-NHR, 2-ketopiperidine-6-carbonyl-His-Pro-NHR and 3-oxoperhydro-1,4-thiazine-5-carbonyl-His-Pro-NHR (R=H, methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, *n*-hexyl, β-phenethyl) were synthesized and pharmacologically tested in the hope of separating the central nervous action from endocrine activity. Among them, γ-butyrolactone-γ-carbonyl-His-Pro-NH_2 was found to have potent central nervous actions in antagonizing pentobarbital sleep and in potentiating apomorphine-induced circling in mice, and only nominal TSH-releasing activity in rats.

Keywords——synthesis of TRH analogs; separation of central nervous action from endocrine activity; γ-butyrolactone-γ-carbonyl-His-Pro-NH_2; antagonism of pentobarbital sleep in mice; potentiation of apomorphine-induced circling in mice; TSH-releasing activity in rats

Thyrotropin-releasing hormone (TRH: L-pyroglutamyl-L-histidyl-L-proline amide), which releases thyrotropin (TSH) as well as prolactin from the anterior pituitary, is widely distributed throughout the central nervous system (CNS),[3)] being concentrated in the nerve terminals.[4)] TRH has also been shown to cause behavioral excitation itself, to potentiate behavioral excitation induced by L-3,4-dihydroxyphenylalanine and to modify behavioral effects of central depressants including barbiturates and ethanol in intact, hypophysectomized or thyroidectomized animals,[5-9)] and was also found to exert some behavioral effects in humans.[10,11)] Such findings have led to the hypothesis that TRH may subserve a non-endocrine function in the CNS, such as synaptic neuromodulation and or neurotransmission. It is also implied that the central nervous action of TRH may be separable from its hypophysiotropic action by chemical modification of the TRH molecule.

1) Amino acids, peptides and their derivatives mentioned in this report are of the L-configuration and γ-butyrolactone-γ-carboxylic acid is of the *S*-configuration unless otherwise mentioned. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2483 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). DCC=dicyclohexylcarbodiimide, HONB=N-hydroxy-5-norbornene-2,3-dicarboxylimide, BSA=bovine serum albumin.

2) Location: *Juso-Honmachi 2-17-85, Yodogawa-ku, Osaka 532, Japan.*

3) M.J. Brownstein, M. Palkovits, J.M. Saavedra, R.M. Bassiri, and R.D. Utiger, *Science*, **185**, 267 (1974).

4) T. Hökfelt, K. Fuxe, O. Johansson, S. Jeffcoate, and N. White, *Europ. J. Pharmacol.*, **34**, 389 (1975).

5) M. Inoue, M. Miyamoto, H. Fujitani, I. Suhara, and Y. Nagawa, *Takeda Kenkyusho Ho*, **35**, 194 (1976).

6) N.P. Plotnikoff, A.J. Prange, Jr., G.R. Breese, M.S. Anderson, and I.C. Wilson, "The Thyroid Axis, Drugs and Behavior," ed. by A.J. Prange, Jr., Raven Press, New York, 1974, pp. 103—113.

7) M. Miyamoto and Y. Nagawa, *Europ. J. Pharmacol.*, **44**, 143 (1977).

8) A.J. Prange, Jr., G.R. Breese, J.M. Cott, B.R. Martin, B.R. Cooper, I.C. Wilson, and N.P. Plotnikoff, *Life Sci.*, **14**, 447 (1974).

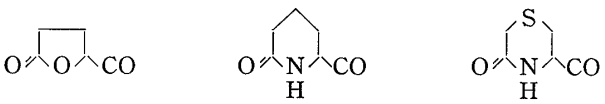
9) M.A. Goujet, P. Simon, R. Chermat, and J.R. Boissier, *Psychopharmacologia* (Berl.), **45**, 87 (1975).

10) A.J. Kastin, R.H. Ehrensing, D.S. Schalch, and M.S. Anderson, *Lancet*, **II**, 740 (1972).

11) I.C. Wilson, A.J. Prange, Jr., P.P. Lara, L.B. Alltop, R.A. Strikeleather, and M.A. Lipton, *Arch. Gen. Psychiatry*, **29**, 15 (1973).

In this study, on the basis of the latter speculation, a number of TRH analogs were synthesized and pharmacologically tested in order to obtain compounds with potent central nervous activity and minimal TSH-releasing activity. The pyroglutamyl moiety of TRH was replaced by a γ-butyrolactone-γ-carbonyl (Blc), 2-ketopiperidine-6-carbonyl (Kpc) or 3-oxoperhydro-1,4-thiazine-5-carbonyl (Otc) group, and the amide group at the C-terminus of these peptide derivatives was also replaced by an alkyl or aralkyl group. Furthermore, a methyl group was introduced into the 3-N position of the imidazole ring of histidine in the Blc group.

Blc Kpc Otc



Materials and Methods

The chemical structures and compound numbers of the peptides synthesized and pharmacologically tested are presented in 3 groups in Table I: group I, Blc-His-Pro-NHR; group II, Kpc-His-Pro-NHR; Group III, Otc-His-Pro-NHR.

Synthesis of the Peptides——The peptide derivatives used here were synthesized by the conventional liquid method. H-His-Pro-NHR (R=H, methyl, *n*-butyl or β-phenethyl) or H-3-Me-His-Pro-NH_2 was coupled with the carboxylic acid (γ-butyrolactone-γ-carboxylic acid,[12] 2-ketopiperidine-6-carboxylic acid[13]

Table I. Chemical Structures and Physicochemical Properties of Synthetic Peptides.

Compound No.[a]	Chemical structure[b]	$[\alpha]_D^{21}$ (in H_2O)	TLC (Rf)[c] A	B	C
Group I					
I-1a	Blc–His–Pro–NH_2 citrate	−45.4°	0.31	0.30	0.31
I-1b	(R)–Blc–His–Pro–NH_2 acetate	−53.3°	0.31	0.30	0.31
I-2	Blc–His–Pro–NHMe acetate	−68.0°	0.36	0.32	0.36
I-3	Blc–His–Pro–NHBu acetate	−66.4°	0.60	0.46	0.57
I-4	Blc–His–Pro–NHPhEt acetate	−78.8°	0.63	0.55	0.60
I-5	Blc–3–Me–His–Pro–NH_2 acetate	−51.1°	0.19	0.29	0.19
Group II					
II-1	Kpc–His–Pro–NH_2	−49.4°	0.24	0.16	0.26
II-2	Kpc–His–Pro–NHBu acetate	−66.1°	0.54	0.32	0.56
Group III					
III-1	Otc–His–Pro–NH_2	−57.5°[d]	0.31	0.22	0.31
III-2	Otc–His–Pro–NHMe acetate	−62.6°	0.33	0.26	0.34
III-3	Otc–His–Pro–NHEt acetate	−67.7°	0.44	0.33	0.47
III-4	Otc–His–Pro–NHPr acetate	−51.6°	0.54	0.37	0.54
III-5	Otc–His–Pro–NHBu acetate	−80.5°	0.59	0.41	0.57
III-6	Otc–His–Pro–NHAm acetate	−65.4°	0.60	0.42	0.60
III-7	Otc–His–Pro–NHHex acetate	−66.4°	0.61	0.45	0.63
III-8	Otc–His–Pro–NHPhEt acetate	−80.5°	0.60	0.45	0.60

a) Satisfactory analytical data were obtained for all products.
b) Me=methyl; Et=ethyl; Pr=*n*-propyl; Bu=*n*-butyl; Am=*n*-amyl; Hex=*n*-hexyl; PhEt=β-phenethyl.
c) The solvent systems used (silica gel) were: A; *n*-butanol: ethyl acetate: acetic acid: H_2O=1:1:1:1, B; dioxane: acetonitrile: H_2O: ethyl acetate: acetic acid=18:12:6:2:1, C; *n*-butanol: acetic acid: H_2O=4:1:1.
d) 25°.

12) A.T. Austin and J. Howard, *J. Chem. Soc.*, **1961**, 3593; C. Eguchi and Y. Kakuta, *Bull. Chem. Soc. Jpn.*, **47**, 1704 (1974).
13) J.P. Greenstein and M. Winitz, "Chemistry of Amino Acids," Vol. 3, 1961, p. 2411.

or 3-oxoperhydro-1,4-thiazine-5-carboxylic acid[14]) by DCC treatment in the presence of pentachlorophenol[15] or HONB[16] to yield the corresponding peptide derivatives except for the compounds III-3,4,6 and 7. These compounds were prepared from Otc-His-Pro-OH and R-NH_2 (R=ethyl, *n*-propyl, *n*-amyl, *n*-hexyl) by DCC treatment in the presence of HONB. The purification was carried out by gel filtration on Sephadex LH-20 using dilute acetic acid as an eluant. The physicochemical properties of the synthetic peptides are listed in Table I.

Pharmacology

1. **Chemicals**——TRH tartrate monohydrate used was synthesized according to the procedure of Hatanaka *et al.*[17] The peptides and agents, except for reserpine (Serpasil®, CIBA), were diluted with 0.9% saline for administration. The doses of the peptides are given in terms of the free form, although most of them were used in a form of an appropriate salt.

2. **Pentobarbital Sleeping Time in Mice**——Groups of 6 or 8 male JCL: ICR mice weighing 20.0—32.5 g were used. Animals were injected *i.v.* with various doses of a test peptide or saline as a control, 10 min after intraperitoneal injection of 55 mg/kg of pentobarbital Na (Mintal®, Tanabe), and the time from the administration of the test peptide to regaining the righting reflex was taken as the sleeping time. Percent shortening of the sleeping time by the test peptide was calculated using the formula: [1-(mean sleeping time in drug-treated group/mean sleeping time in saline-treated control group] $\times 100$. Statistical significance between drug-treated and control group was determined by means of Student's *t*-test.

3. **Apomorphine-Induced Circling Behavior in Mice with Unilateral Caudate Lesion**——This test was carried out using the method described by Fukuda *et al.*[18] Male JCL: ICR mice weighing 28.0—35.0 g were fixed on a stereotaxic instrument under light ether anesthesia, and an injection needle, with 0.8 mm outer and 0.6 mm inner diameters, was inserted unilaterally into the caudate nucleus 3.5 mm in depth through a small burr hole made on the skull 3 mm posterior and 2 mm lateral to the bregma. The unilateral caudate nucleus was partially lesioned by aspirating the brain tissue with a suction pump connected to the intracerebral needle, according to the method of Lotti.[19] Two weeks following surgery, the animals exhibiting a clear 360° turn toward the lesioned side (ipsilateral circling) in response to intraperitoneal injection of 2 mg/kg of apomorphine HCl (August Brandes) were selected and employed for drug testing repeatedly at weekly intervals for several weeks, beginning one week after selection.

In the drug testing, animals treated with 2 mg/kg of reserpine *i.p.* 18—22 hr prior to test were placed individually in 1 liter glass beakers. Thirty min later they received an intraperitoneal injection of test peptide or saline as a control, followed by an intraperitoneal administration of 0.25 mg/kg of apomorphine a further 30 min later. The ipsilateral circlings were counted for 1 min each at 10, 20 and 30 min after administration of apomorphine, and the total counts were averaged from a group of 8—16 animals. Potentiation of the apomorphine circling by the test peptide was recorded as the ratio of the mean count in the drug-treated group to that in the control group. Statistical comparison between drug-treated and control groups was done by means of Student's *t*-test.

4. **TSH-Releasing Activity in Rats**——Male Sprague-Dawley (JCL: SD) rats weighing 200—230 g at 6 weeks of age were intravenously administered with various doses of a test peptide *via* the tail vein. The test peptide was dissolved in 0.9% saline containing 0.1% BSA (Wako Pure Chemical), and administered in a volume of 0.5 ml per animal. Control animals received the BSA-containing saline alone. Fifteen min after the administration, blood was taken from the abdominal aorta under urethane anesthesia (800 mg/kg, *i.p.*). Sera were separated as quickly as possible, and stored at −20° until assays. Serum TSH levels were determined by the double-antibody radioimmunoassay method of Midgley[20] using the NIAMDD-Rat-TSH kit. Rat-TSH was labelled with ^{131}I according to the chloramine-T method of Hunter and Greenwood.[21] Results were expressed in terms of NIAMDD-Rat-TSH RP-1, and the TSH-releasing potency of the test peptides was calculated by the parallel line assay method.[22]

14) H. Ozawa, *Bull. Chem. Soc. Jpn.*, **36**, 920 (1963).
15) G. Kupryszewski and M. Kaczmarek, *Rocz. Chem.*, **35**, 931 (1961).
16) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.*, **22**, 1857 (1974).
17) C. Hatanaka, M. Obayashi, O. Nishimura, N. Toukai, and M. Fujino, *Biochem. Biophys. Res. Commun.*, **60**, 1345 (1974).
18) N. Fukuda, M. Miyamoto, S. Narumi, Y. Nagai, T. Shima, and Y. Nagawa, *Nippon Yakurigaku Zasshi*, **75**, 251 (1979).
19) V.J. Lotti, *Life Sci.*, **10**, 781 (1971).
20) R. Midgley, Jr., *Endocrinology*, **79**, 10 (1966).
21) W.M. Hunter and F.C. Greenwood, *Nature* (London), **194**, 495 (1962).
22) D.J. Finney, "Statistical Method in Biological Assay," 2nd ed. Charles Griffin and Co., Ltd., London, 1964, p. 99.

Table II. Central Nervous System Activities

Compound No.	Antagonism of pentobarbital sleep				Potentiation of apomorphine circling			
	Dose[a] mg/kg *i.v.*	N[b]	% Shortening	MED[c] mg/kg *i.v.*	Dose mg/kg *i.p.*	N	Potentiation ratio	MED mg/kg *i.p.*
TRH	0.125	8	19.8	0.25	1.25	8	1.44	2.5
	0.25	8	38.1*		2.5	8	5.00**	
	0.5	8	52.1***		5.0	8	6.67***	
	1.0	8	43.5**		10.0	8	6.33**	
I-1a	0.02	8	14.2	0.04	0.41	10	2.27	0.82
	0.04	8	30.9*		0.82	10	2.61**	
	0.08	8	42.7**		1.64	10	5.62**	
	0.16	8	43.3**		3.27	10	6.31**	
I-1b	0.21	8	4.3	0.41	8.3	10	1.94	>8.3
	0.41	8	27.6**					
	0.83	8	27.6**					
I-2	0.94	6	10.2	>0.94	9.4	8	0.31	>9.4
I-3	0.93	6	36.4	>0.93	9.3	8	2.33	>9.3
I-4	0.93	6	0	>0.93	9.3	8	1.33	>9.3
I-5	0.06	8	16.5	0.11	0.28	10	1.94	0.55
	0.11	8	27.4**		0.55	10	4.50**	
	0.22	8	47.0***		2.2	10	5.00***	
	0.43	8	63.0***					
	0.86	8	64.4***					
II-1	0.25	6	18.2	0.5	2.5	8	1.88	5.0
	0.5	6	29.3*		5.0	8	4.56*	
	1.0	6	66.5***					
II-2	0.44	6	13.8	1.76	2.2	8	1.92	4.4
	0.88	6	18.9		4.4	8	4.00**	
	1.76	6	43.6*					
III-1	0.125	8	50.7***	<0.125	1.25	8	1.63	2.5
	0.25	8	48.4***		2.5	8	3.50***	
	0.5	8	53.2***		5.0	8	3.63***	
	1.0	8	66.5***		10.0	8	6.56***	
III-2	0.22	6	4.3	0.44	4.4	8	1.25	8.8
	0.44	6	31.1*		8.8	8	8.80*	
	0.88	6	65.3***					
III-3	0.22	6	41.1*	<0.22	8.8	8	2.38	>8.8
	0.44	6	45.1**					
	0.88	6	45.6**					
III-4	0.11	6	−9.6	0.22	8.8	8	2.00	>8.8
	0.22	6	43.8*					
	0.44	6	45.6**					
	0.88	6	44.8**					
III-5	0.11	8	15.5	0.22	0.55	8	1.36	1.1
	0.22	8	41.8*		1.1	16	2.36*	
	0.44	8	35.0*		2.2	8	3.00**	
	0.88	8	49.0**		4.4	8	3.64***	
III-6	0.45	6	27.7	0.90	9.0	8	1.54	>9.0
	0.90	6	28.8*					
III-7	0.22	6	17.0	0.90	9.0	8	1.60	>9.0
	0.45	6	32.0					
	0.90	6	48.8**					
III-8	0.90	6	20.2	>0.90	9.0	8	1.29	>9.0

a) Doses are expressed in terms of the free drug.
b) Number of animals used.
c) Minimum dose producing a significant effect.
Statistical significance *vs.* control. *p <0.05, **p <0.01, ***p <0.001.

Results and Discussion

Central Nervous Activity

Antagonism of pentobarbital sleep and potentiation of the apomorphine circling in mice are the most characteristic pharmacological properties of TRH on the CNS, and the latter action has been postulated to indicate a facilitation of dopaminergic transmission at the post-synaptic level under supersensitization of the dopamine receptors in the striatum.[18]

Table II shows the central activities of the peptides, compared with those of TRH. There seemed to be no parallel between alterations in antagonistic activity on pentobarbital sleep and in potentiating activity of apomorphine circling upon chemical modification of the TRH molecule, suggesting that these effects may be independent of each other. The central activities of the peptides were compared on the basis of the minimum effective doses, because the dose-response relationships were not necessarily parallel.

Compound I-1a, in which the pGlu moiety of the TRH molecule was replaced by an (*S*)-Blc group, showed the highest central activity; it was approx. 6 times as active as TRH in shortening the pentobarbital sleeping time and approx. 3 times as active as TRH in potentiating the apomorphine circling. The (*R*)-isomer with the (*R*)-Blc group (compound I-1b) was, however, much less active than the (*S*)-isomer. In the (*S*)-Blc group, introduction of a methyl group at the 3-N position of the imidazole ring of histidine (compound I-5) did not reduce the high central activity. Replacement with an Otc group resulted in a marked increase in the pentobarbital antagonism without altering the apomorphine potentiating activity (compound III-1 *vs.* TRH). The Kpc group (compound II-1) reduced both activities to approx. half those of TRH.

Introduction into the Pro-NH_2 of an Me, Bu or PhEt group in group I and of an Am, Hex or PhEt group in group III produced an almost complete loss of both central activities (compounds I-2, -3, -4, III-6, -7, -8). In group III, the introduction of an Me, Et or Pr group did not affect the pentobarbital antagonism activity but greatly reduced or almost completely removed the apomorphine potentiating activity (compounds III-2, -3, -4 *vs.* III-1). The Bu group in groups II and III tended to increase the apomorphine potentiating activity with a slight reduction in antipentobarbital activity (compound II-2 *vs.* II-1; III-5 *vs.* III-1).

TSH-Releasing Activity

Of 5 peptides recognized to be potent in both central activity tests, compounds I-1a, I-5, III-1 and III-5 were selected for the assay of TSH-releasing activity, in comparison with TRH. Since all these peptides were found, in a preliminary study, to produce a peak serum TSH level at approximately 15 min after a single intravenous injection, the potencies were assessed from the relationship between doses and serum TSH levels obtained at this time. As shown in Table III, compound I-1a and TRH caused a dose-dependent elevation in the serum TSH levels, and the responses were linearly related to the logarithm of the doses. Both regression lines were statistically parallel, and the potency ratio of compound I-1a relative to TRH[23] was estimated to be 0.024. Similarly, the regression lines obtained with compounds I-5, III-1, III-5 and TRH were all parallel, and the potency ratios of compounds I-5, III-1 and III-5 relative to TRH were calculated to be 0.196, 1.58 and 1.19, respectively. Thus, replacement of the pGlu moiety with Blc remarkably reduces the TSH-releasing activity to approx. 1/40 that of TRH, in contrast to the marked enhancement in the central activity. In the case of the Blc group, however, the TSH-releasing activity became approx. 10 times more potent than that of compound I-1a upon the introduction of a methyl group at the 3-N position of the imidazole ring of histidine. Replacement with Otc seemed to result in a slight enhancement of the TSH-releasing activity.

23) M. Shikata, T. Masaki, S. Akashi, and R. Nakayama, *Yakuri To Chiryo*, **4**, 1076 (1976).

Table III. TSH-Releasing Activity

Exp. No.	Compound No.	Dose[a] μg/rat *i.v.*	N[b]	Serum TSH[c] (mean±S.E.M.) μg/100 ml	Potency ratio relative to TRH (95% confidence limits)
I	TRH	0.03125	8	37±9	1.0
		0.125	8	103±16	
		0.5	8	237±23	
	I-1a	2	8	61±9	0.024
		8	8	158±16	(0.017—0.036)
		32	8	246±17	
II	TRH	0.1	8	118±10	1.0
		0.5	8	252±21	
	I-5	0.2	8	53±6	0.196
		1	8	153±14	(0.134—0.285)
		5	8	316±23	
III	TRH	0.125	9	105±9	1.0
		0.5	9	168±11	
	III-1	0.0625	9	80±6	1.58
		0.25	8	169±13	(1.04—2.30)
	III-5	0.22	9	140±12	1.19
		0.88	8	198±8	(0.74—2.31)

a) Doses are expressed in terms of the free drug.
b) Number of animals used.
c) Vehicle control: 9±2 μg/100 ml (N=8) in Exp. I;
24±5 μg/100 ml (N=8) in Exp. II;
7±3 μg/100 ml (N=3) in Exp. III.

There are a few reports regarding a correlation between central action and TSH-releasing action of TRH analogs. In shaking behavior induced by intraventricular injection in rats, N^{3-im}-methyl TRH is approx. 10 times as potent as TRH, whereas N^{1-im}-methyl TRH is approx. 10 times less potent than TRH.[24] These potencies parallel the TSH-releasing activities.[25] These investigators suggested that it may be difficult to separate chemically the behavioral properties. The present study revealed that N^{3-im}-methylation in Blc-His-Pro-NH_2 greatly increased the TSH-releasing activity without altering the central nervous activity. A novel TRH analog, L-N-(2-oxopiperidine-6-carbonyl)-L-histidyl-L-thiazolidine-4-carboxamide (MK-771) has recently been reported to be equipotent with TRH in its hypophysiotropic action, but approx. 100 times as potent as TRH in shortening the pentobarbital sleeping time in normal mice and approx. 35 times as potent as TRH in restoration of the methazolamide electroshock anticonvulsant activity in reserpine-treated mice.[26,27] The compound I-1a obtained in the present study is superior to MK-771 in respect to separation of the central activity from the hypophysiotropic action, because the former compound is approx. 40 times less potent than TRH in the TSH-releasing activity and 3—6 times more potent than TRH in the central activity. Further pharmacological studies on the CNS actions of this compound will be conducted.

Acknowledgement The authors wish to express their cordial thanks to Dr. A.F. Parlow for providing the Rat-TSH kit under the NIAMDD-Rat Pituitary Hormone Distribution Program.

24) E. Wei, H. Loh, and E.L. Way, *Europ. J. Pharmacol.*, **36**, 227 (1976).
25) W. Vale, G. Grant, and R. Guillemin, "Frontiers in Neuroendocrinology," ed. by W.F. Ganong and L. Martini, Oxford University Press, New York, 1973, p. 375.
26) D.F. Veber, F.W. Holly, S.L. Varga, R. Hirschman, R.F. Nutt, V.J. Lotti, and C.C. Porter, "Peptides 1976," Proceedings of the 14th European Peptide Symposium, 1977, p. 453.
27) C.C. Porter, V.J. Lotti, and M.J. De Felice, *Life Sci.*, **21**, 811 (1977).