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SYNTHESIS AND BIOLOGICAL ACTIVITY OF 5-FLUOROIMIDAZOLE-TRH

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The 5-fluoroimidazole analogue of thyrotropin-releasing hormone, obtained by total synthesis from 5-fluoro-L-histidine, neither binds to rat pituitary cells nor stimulates release of prolactin from them. Lévine-Pinto et. al. reported an agonist, which was generated during presumptive photofluorination of the hormone and which they believed to be the 5-fluoro analogue. In addition to the striking contrast in biological activities, the chemical properties of the agonist differ markedly from those of our peptide and are inconsistent with expectation for the fluoroimidazole moiety. Despite its inactivity in pituitary functions, the authentic 5-fluoro analogue mimics the natural hormone with respect to cardiovascular responses in the central nervous system.

The synthesis of 5-fluoro-L-histidine**, by photochemical decomposition of the 5-diazonium fluoroborate, was first reported by Kirk and Cohen in 1971 (1-3). In a subsequent progress report (4), these authors noted that the 5-fluoroimidazole analogue of TRH had been synthesized from 5-fluoro-L-histidine, and that the analogue had been found essentially inactive as an agonist or antagonist of TRH with respect to its pituitary activities; accordingly, the study was terminated before sufficient data had been compiled for publication. More recently, an alternative synthesis of the same TRH analogue was reported (5), in which the elaboration of the 5-aminoimidazole moiety and the photochemical introduction of fluorine were performed on TRH itself. This work was done, apparently, to demonstrate

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**Tautomer preference is unknown and the assignment is arbitrary.
Abbreviations: TRH = thyrotropin-releasing hormone; Im = imidazole; DMEM = Dulbecco's modified Eagle's medium.

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the feasibility of specific fluorination of histidine at the polypeptide level. In contrast with our earlier results, the latter analogue was found to be as active as TRH in short-term assays of prolactin release and 4.5 times more active in long-term assays.

Certain results in the latter report (5) seemed inconsistent with our own experience in amino- and fluoroimidazole chemistry: (a) despite the notorious instability of 5-aminoimidazoles (3,6,7), the presumed 5-amino-Im-TRH was purified on both Dowex and silica gel columns; (b) amino acid analysis of the fluorinated peptide, which was reported to give only equivalent amounts of glutamic acid and proline, should also have given a peak near valine for 5-fluorohistidine; (c) mass spectrometry should have given fragments 18 units heavier than those derived from the parent peptide—not 19, as reported; (d) the pK_1 value reported for 5-fluoro-Im-TRH (5.7) is considerably higher than the values found for any other 5-fluoroimidazoles (1.5-3.0) (8), and is totally inconsistent with expectation for any halogenated imidazole; (e) a peptide with the claimed pK_1 value should show electrophoretic mobility close to that of TRH itself (pK_1 6.2) (9)—yet, the implication from Table I of the report (5) is that the fluoro analogue failed to migrate; (f) the report gives neither analytical nor ^{19}F NMR data to support the presence of fluorine in the compound. In the light of these puzzling results, we have once again synthesized 5-fluoro-Im-TRH from authentic 5-fluoro-L-histidine (3) and have again evaluated its ability to stimulate prolactin release.

MATERIALS AND METHODS

5-Fluoro-Im-TRH***: 5-Fluoro-Im-L-histidine (3) was condensed with pentachlorophenyl L-pyroglutamate (Sigma Chemical Co.) in dimethylformamide in the presence of triethylamine (88% yield); the resulting dipeptide acid was coupled with L-prolineamide in dimethylformamide by use of dicyclohexylcarbodiimide and hydroxybenztriazole (45% yield). The tripeptide was purified by ion exchange on Amberlite IR-4B, by preparative TLC on silica gel and, finally, by column chromatography on silica gel. The homogeneity of the product was verified by TLC in three solvent systems, by HPLC, and by ^1H and ^{19}F NMR spectroscopy. The identity of the product was demonstrated by elemental analysis, by mass spectrometry (CI, CH_4), 381 (M+1), 253 (cyclo-5-fluoro-Im-His-Pro + 1), and by amino acid analysis—Glu (1), fluoro-

***Full experimental details will be published separately.

His (0.7), Pro (1). 5-FluoroHis follows and overlaps valine; the free amino acid is degraded to about the same extend under the same hydrolysis conditions. The hydrated peptide gives mp 166-170 °C, decomposing at 205-209 °C; $[\alpha]_D^{24}$ -55.3° (c 0.7, H_2O); ^{19}F NMR (CD_3OD) δ -68.2 ppm (upfield from CF_3COOH).

Cell Culture: GH_4C_1 cells were grown in an atmosphere of 95% air and 5% CO_2, in a 1/1 mixture of DMEM and Ham's Nutrient Mixture F10 supplemented with 15% horse serum, 300 nM insulin and 1 nM 17β-estradiol (10). Cultures were plated at an initial density of 1 x 10^4 cells per 35 mm well for measurements of prolactin release and at 4 x 10^4 cells per 35 mm well for binding studies. The medium was changed 3 and 6 days after plating, and the experiment was begun 7 days after plating.

Binding Studies: L-[2,3,4,5-^{3}H]Proline-TRH, 100 curies/mmol, was obtained from New England Nuclear Co. Medium was removed and replaced with fresh medium containing 5 nM [^{3}H]TRH plus the indicated concentrations of unlabeled peptide. After 1 hour, cells were rinsed, solubilized and counted as previously described (10).

Effects on Prolactin Release and Synthesis: Culture medium was removed and replaced with fresh medium containing the indicated concentrations of peptide. Medium was collected 2 hours later to measure effects on prolactin release and 48 hours later to measure effects on prolactin synthesis. Prolactin was assayed by microcomplement fixation, as previously described (11).

RESULTS AND DISCUSSION

The synthetic sequence provided a homogeneous product whose purity and identity were demonstrated by seven criteria (see Materials and Methods). Careful titration of the ring-fluorinated peptide (in both directions) gave values of pK_1 = 1.94 ± 0.02 and pK_2 = 11.79 ± 0.11. These values are wholly consistent with those obtained for simpler 5-fluoroimidazoles (8). As further confirmation, α-N-acetyl-5-fluoro-L-histidine methyl ester (3) was found to have pK_1 = 2.10 ± 0.03.

As shown in Figure 1, 5-fluoro-Im-TRH fails to effect even a modest displacement of [^{3}H]TRH from loaded rat pituitary cells. The product obtained by Lévine-Pinto et. al. (5) showed significant binding only at concentrations as high as 10^{-6} M; our peptide showed no measurable binding even at this level. Furthermore, the data of Table 1 show that authentic 5-fluoro-Im-TRH does not stimulate prolactin release in vitro, either in short or long-term assays. These new results are consistent with our earlier claims (4) and stand in direct contrast to those reported by the Paris group. The potent analogue obtained in the latter work is clearly not 5-fluoro-Im-TRH, and we are presently involved in investigating its identity.

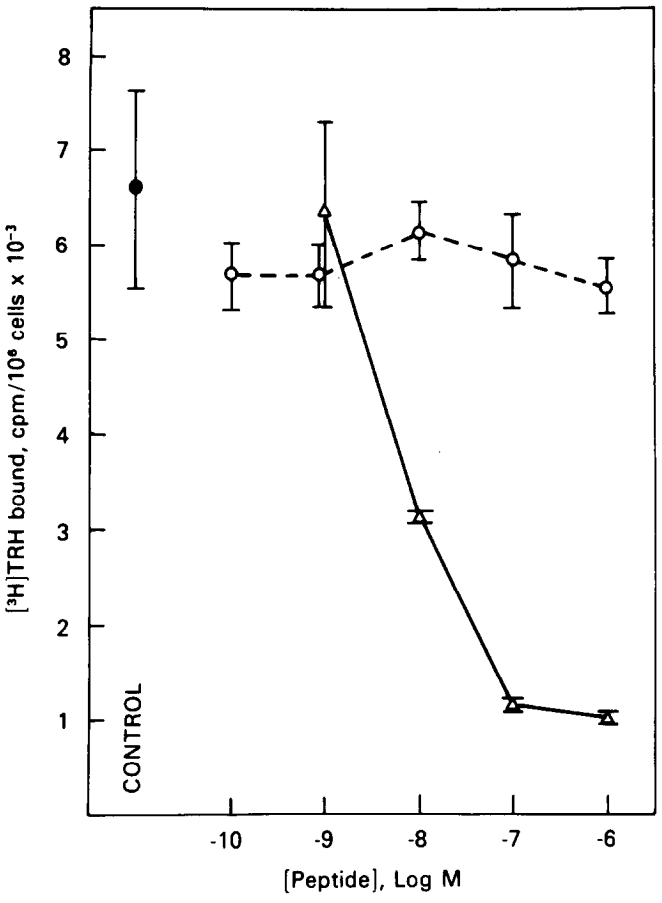

Figure 1. Residual radioactivity in GH_4C_1 rat pituitary cells, following additon of various concentrations of TRH (Δ) and of 5-fluoro-Im-TRH (O). Each point is the mean of triplicate plates and the bars indicate the standard deviation.

At the time of the earlier evaluation of the fluoro analogue (1971), the widespread CNS activities of TRH (12,13) had not yet been fully recognized. Our current studies (14) reveal that the fluoroimidazole

Table 1. Effects of TRH and of 5-Fluoro-Im-TRH on Prolactin Content of Medium

Peptide	Prolactin, μg/10^5 cells		
	2 hrs	48 hrs (Run 1)	48 hours (Run 2)
Control	0.67 ± 0.04	8.3 ± 0.6	4.9 ± 1.1
TRH (5 nM)	1.18 ± 0.16		10.4 ± 2.3
(10 nM)		14.8 ± 1.5	
(100 nM)		23.0 ± 0.6	13.1 ± 1.6
(1000 nM)	1.54 ± 0.20		15.0 ± 2.0
5-F-Im-TRH (5nM)	0.59 ± 0.05	6.8 ± 1.0	5.1 ± 0.5
(100 nM)		7.1 ± 0.6	
(1000 nM)	0.53 ± 0.01		7.3 ± 2.4

Each 2-hr value is the mean of duplicate plates ± the range and each 48-hr value is the mean of triplicate plates ± the standard deviation.

analogue is almost as effective as TRH in increasing blood presure and heart rate, following microinjection into the rat hypothalamus. To our knowledge, 5-fluoro-Im-TRH may be the first analogue which achieves essentially total separation of pituitary and CNS activities (15).

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