Biochemical Pharmacology, Vol. 29, pp. 2619–2623.

0006-2952/80/1001-2619 $02.00/0

AN ANALOGUE OF THYROTROPIN RELEASING HORMONE WITH IMPROVED BIOLOGICAL STABILITY BOTH *IN VITRO* AND *IN VIVO*

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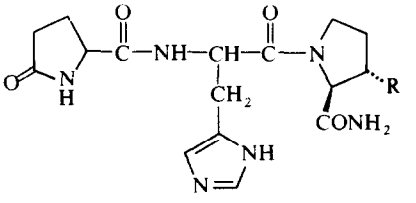
(*Received* 6 *March* 1980; *accepted* 1 *May* 1980)

Abstract—The metabolism of TRH and a *trans*-3-methyl-prolineamide analogue have been studied both *in vitro* and *in vivo* using a rat model. The rates of degradation of the two tripeptides have been compared. Studies *in vitro* using both plasma and brain homogenates showed the analogue to have increased biological stability and show qualitative differences in metabolism as compared to TRH. Studies *in vivo* related to those observed *in vitro*. The increased biological stability of the TRH analogue could explain its improved CNS potency *in vivo*.

The hypothalamic releasing factor thyrotropin releasing hormone (TRH, L-pyroglutamyl-L-histidyl-L-prolineamide) increases the output of thyroid stimulating hormone (TSH) from the pituitary [1, 2]. A stimulation of the release of prolactin and growth hormone has also been observed [3, 4]. Following the characterization and synthesis of TRH in amounts sufficient for pharmacological evaluation, a number of reports have suggested that TRH exerts direct actions on the central nervous system (CNS). Rapid short lasting anti-depressant activity in man was described for TRH by Prange, Wilson and co-workers [5, 6] and confirmed by others [7, 8]. Of a range of synthetic analogues of TRH prepared in these laboratories certain compounds have shown increased CNS potency and duration of action as compared to that of the native tripeptide. Pharmacological evaluation of one such compound, L-pyroglutamyl - L - histidyl - L - (trans - 3 - methyl)prolineamide, (3-Me-Pro)TRH, showed this peptide to be four times as potent as TRH in the reversal of reserpine-induced hypothermia in mice.*

It is becoming increasingly apparent that a wide range of peptide hormones are subject to hydrolytic degradation by enzymes present in serum and various other tissues. The enzymatic hydrolysis of luteinizing hormone releasing hormone (LRH), oxytocin and the kinins has already been documented [9–11] and more recently the enzymic inactivation of enkephalins by plasma and brain tissue has been reported [12]. The hydrolysis of TRH has also attracted attention, reports indicating that TRH is rapidly degraded and inactivated by plasma and a variety of animal tissues *in vitro* [13–17]. It was considered that the stability to enzymatic degradation of the analogue as compared to the natural peptide may be an influencing factor in the increased pharmacological potency of the compound and as such the metabolism and rates of degradation of TRH and (3-Me-Pro) TRH have been investigated and compared both *in vitro* and *in vivo*.



Structures of TRH (R = H) and (3-Me-Pro)TRH (R = Me)

MATERIALS AND METHODS

Materials

Both [2-^{14}C-histidyl]TRH and [2-^{14}C-histidyl]-(3-Me-Pro)TRH (sp. act. 3.74 μCi . mg^{-1} and 3.60 μCi . mg^{-1}, respectively) were synthesized from [ring-2-^{14}C] histidine (The Radiochemical Centre, Amersham, U.K.) by the method of Morgan.†

Authentic samples of L-histidyl-L-prolineamide (His-Pro-NH_2) and L-histidyl-L-*trans*-3-methylprolineamide (His-3-Me-Pro-NH_2) were converted to their corresponding diketopiperazines His-Pro and His-(3-Me)-Pro, respectively, by spontaneous cyclization of the amide hydrobromides in excess 50% (v/v) aqueous pyridine overnight at 20°.

Animals

Male Sprague–Dawley rats (Bantin & Kingman Ltd., Grimston, N. Humberside, U.K., 220–250 g) were used in all studies. Blood was obtained by cardiac puncture under light ether anaesthesia and plasma was separated by centrifugation.

Thin-layer chromatography

T.l.c. was carried out on 20 × 20 cm silica F_{254} plates (Merck Ltd., Darmstadt, F.R.G.). Three sol-

* Brewster, Dettmar, Lynn, Morgan, Rance and Metcalf, in preparation.

† B. A. Morgan, unpublished work.

vent systems were used: (a) chloroform–methanol–NH_4OH (sp. gr. 0.88), (5:3:1, v/v); (b) chloroform–methanol–NH_4OH (sp. gr. 0.88), (30:18:1, v/v); (c) ethyl acetate–methanol–NH_4OH (sp. gr. 0.88), (10:4:1, v/v). Authentic marker amino acids and peptides were visualized with Pauly's reagent or Ninhydrin.

Quantitative determination of metabolites

Both unchanged substrates and metabolites from *in vitro* or *in vivo* samples were analysed by quantitative radioassay after separation by t.l.c. Samples prepared as described below were applied as 2–5 cm bands and dried thoroughly before development. Authentic markers of potential metabolites were also chromatographed. Developed chromatograms were divided into 1 cm zones, the silica removed and suspended in 2 cm^3 water and 5 cm^3 of scintillation medium comprised of toluene–Triton X-100 (2:1, v/v) containing 0.75% (w/v) of butyl PBD (Koch-Light Ltd., Colnbrook, U.K.). Prepared samples were counted for ^{14}C in an Intertechnique SL 4221 liquid scintillation counter. Since the products generated by the Pauly reagent are photon quenching agents, sample chromatograms were not themselves sprayed but were carefully masked whilst adjacent authentic standards (co-chromatographed with duplicate sample) were visualized. For determination of total plasma radioactivity, aliquots of plasma (100 μl) were added to an aliquot (10 cm^3) of the scintillant described above together with water (1 cm^3). Dpm values were obtained using an on-line, linear interpolation, automatic external standard channels ratio programme (Intertechnique Ltd.). The presence of histidine was investigated using a standard reverse isotope dilution technique, histidine as diluter being recrystallized from aqueous methanol.

Studies in vitro

Degradation of [^{14}C]*TRH and* [^{14}C]-(3-*Me-Pro*)*TRH by rat plasma.* A 50% (v/v) solution of fresh rat plasma in phosphate buffer (100 mM, pH 7.4) was incubated at 37° in the presence of either [^{14}C]TRH or [^{14}C]-(3-Me-Pro)TRH at a concentration of 4 $\mu g \cdot cm^{-3}$. Incubations were begun by the addition of substrate and then aliquots (200 μl) were removed at various time intervals over 2 hr. Samples were treated with ice-cold methanol (100 μl) and precipitated protein centrifuged using an Eppendorf 3200 bench centrifuge. Samples were stored on ice prior to t.l.c. and subsequent radioassay as described earlier. Control incubations were carried out in 100 mM phosphate buffer, pH 7.4.

Degradation of [^{14}C]-*TRH and* [^{14}C]-(3-*Me-Pro*)*TRH by rat brain homogenates.* Rats were killed by cervical fracture, brains were removed and rinsed free of blood in 0.32 M sucrose. Homogenates (5% w/v) were prepared in 100 mM phosphate buffer, pH 7.4, using two strokes of a teflon pestle power driven at 1000 rpm. Incubations were begun by the addition of substrate to a concentration of 5 $\mu g \cdot cm^{-3}$. Aliquots (200 μl) were removed, treated with methanol (100 μl) and analysed as described in the plasma experiments. Suitable control incubations were carried out in phosphate buffer alone.

Studies in vivo

At various times after intravenous dosing with either [^{14}C]TRH or [^{14}C]-(3-Me-Pro)TRH (10 mg/kg), individual rats were heart bled under light ether anaesthesia, then killed by cervical fracture. Blood samples were transferred to heparinized tubes and immediately cooled in ice–water before centrifugation at 4°. Care was taken to ensure that samples always remained at <10° until analysed. Plasma samples were assayed for total radioactivity, then analysed by quantitative radioassay of thin-layer chromatograms as described earlier.

Identification of the major plasma metabolite of (3-*Me-Pro*)*TRH*

A total of 100 cm^3 of rat plasma in 100 cm^3 0.1 M phosphate buffer, pH 7.4, and containing [^{14}C]-(3-Me-Pro)TRH (8 $\mu g \cdot cm^{-3}$) was incubated at 37° for 18 hr. Methanol (100 cm^3) was added, precipitated protein removed by centrifugation and the supernatant fraction concentrated by evaporation and then freeze-dried. The solid residue was extracted with methanol (4 × 25 cm^3) and the concentrated methanolic extracts subjected to chromatography on a silica column (Kieselgel 60, 50 × 2.0 cm) using solvent system (b). Eluted fractions (15 cm^3) were monitored for radioactivity and the required fractions combined and evaporated. The metabolite, radiochemically pure by t.l.c., was then further purified in sequential fashion by t.l.c. (as a 5 cm band), first in system (a), then system (c). Material was recovered from the silica in each case by elution with small volumes of Analar methanol. A small portion of the final product was prepared for direct insertion mass spectrometry according to the method of Rix *et al.* [18].

Mass spectrometry

Direct insertion mass spectra were recorded on an LKB 2091 mass spectrometer operating at 70 eV and an inlet temperature of 100°.

RESULTS

Studies in vitro. The *in vitro* incubation of [^{14}C]TRH or [^{14}C]-(3-Me-Pro)TRH in rat plasma led to a degradation of both peptides, the products of which were readily separated by t.l.c. in system (a) (see Fig. 1). In the case of TRH, histidine appeared as the major degradation product and this was confirmed by isotope dilution and recrystallization to constant specific radioactivity. There was little evidence to indicate the presence of significant amounts of TRH-acid(L-pyroglutamyl-L-histidyl-L-proline) nor the L-histidyl-L-proline diketopiperazine (His-Pro) which can arise by cyclization of the unstable dipeptide L-histidyl-L-prolineamide (His-Pro . NH_2) [13, 17]. The metabolism of (3-Me-Pro)TRH was in marked contrast to the above, however; there being only small amounts of histidine but larger quantities of a high R_f component (Fig. 1) which co-chromatographed with an authentic sample of the corresponding diketopiperazine His-(3-Me)-Pro.

The metabolism of TRH and (3-Me-Pro)TRH by brain homogenates was similar to that observed in

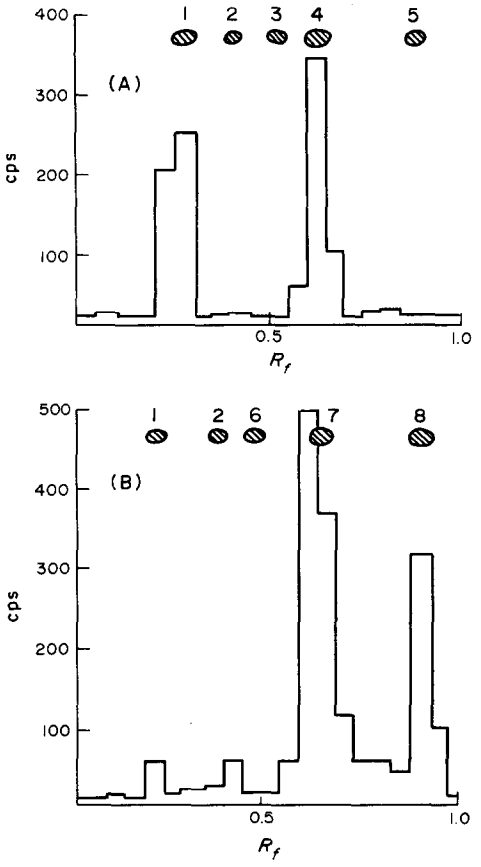


Fig. 1. Metabolic patterns after *in vitro* incubation of [^{14}C]TRH or [^{14}C] (3-Me-Pro)TRH in rat plasma. (A) TRH incubation after 8 min; (B) (3-Me-Pro)TRH incubation after 2 hr. Positions of authentic co-chromatographed marker compounds are shown on the chromatograms as 1, L-histidine; 2, L-pyroglutamyl-L-histidine; 3,pyroglutamyl-L-histidyl-L-proline (TRH-acid); 4, TRH; 5, His-Pro; 6, L-pyroglutamyl-L-histidyl-L-3-methylproline[(3-Me-Pro)TRH-acid]; 7, (3-Me-Pro)TRH; 8, His-(3-Me)-Pro. All incubations were carried out at an initial peptide concentration of 4 μg . cm^{-3}.

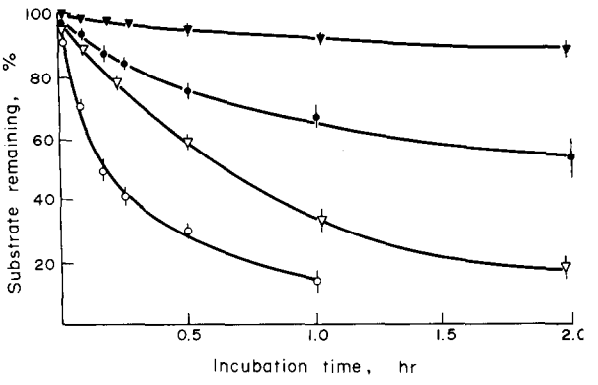


Fig. 2. Degradation of TRH or (3-Me-Pro)TRH by rat plasma or rat brain homogenates. The [^{14}C-His]-labelled peptides were incubated at a concentration of 4 μg . cm^{-3} (plasma) or 5 μg . cm^{-3} (brain). ○, TRH in plasma; ●, (3-Me-Pro)TRH in plasma; ▽, TRH in 5% brain homogenate; ▼, (3-Me-Pro)TRH in 5% brain homogenate. All points represent the mean of five separate determinations ± S.D. as indicated by the vertical bars.

plasma. T.l.c. showed TRH to be rapidly hydrolysed to free histidine and (3-Me-Pro)TRH to be slowly converted to a component which co-chromatographed with the authentic diketopiperazine His-(3-Me)-Pro. Incubation of each peptide in a similar manner with hypothalamus homogenates gave similar metabolic patterns and hydrolysis rates to those obtained using whole brain.

Kinetic studies on the degradation of the two peptides by plasma and brain showed striking differences between their rates of hydrolysis (see Fig. 2). Although TRH was rapidly degraded by both tissues, (3-Me-Pro)TRH was hydrolysed relatively slowly. Semi-logarithmic plots of the hydrolysis curves showed the reactions to follow essentially first order kinetics, the half-life of TRH in plasma and brain being 12 and 30 min, respectively. In the case of (3-Me-Pro)TRH the half-life in plasma was 110 min whereas in brain only a 15 per cent change occurred within 120 min.

Confirmation of the identity of the major metabolite of (3-Me-Pro)TRH produced by plasma and brain was obtained by electron impact mass spectrometry of the material purified from a separate incubation carried out in rat plasma. The spectrum (Fig. 3) was identical to that of authentic synthetic His-(3-Me)-Pro, principal fragment ions occurring at m/e 84, 82, 168, 248 (M^+) and 81 in each spectrum. Incubation of the purified radiolabelled metabolite in rat plasma (8 μg . cm^{-3}) at 37° showed no change after a total of 3.5 hr. Control incubations of both peptides in phosphate buffer alone showed that the degree of chemical hydrolysis of both peptides was less than 2 per cent over a 5 hr period.

Studies in vivo. Following intravenous administration of [^{14}C]TRH or [^{14}C]-(3-Me-Pro)TRH, a rapid elimination of radioactivity from plasma was observed, the rates being very similar for both peptides (see Fig. 4). Radioassay of plasma samples after t.l.c. showed a similar metabolite pattern to the *in vitro* situation in that TRH was extensively degraded to histidine (and presumably unlabelled pyroglutamic acid and proline), whereas in the case of (3-Me-Pro)TRH, only small amounts of His-(3-Me)-Pro and some histidine were apparent, most of the radioactivity being present as unchanged peptide. Quantitative analysis of the above chromatograms confirmed that the relative proportions of unchanged substrate were considerably higher in the case of the (3-Me-Pro)TRH samples (see Fig. 5). Since after intravenous administration total drug related material is cleared from the systemic circulation at similar rates for each peptide (Fig. 4), improved peptide stability must necessarily result in higher absolute plasma concentrations of (3-Me-Pro)TRH over TRH at the times examined.

DISCUSSION

It is clear that a number of different peptide hormones undergo rapid degradation in mammalian tissues [9–17]. The present results have supported evidence that thyrotropin releasing hormone (TRH) is rapidly degraded by plasma and other mammalian

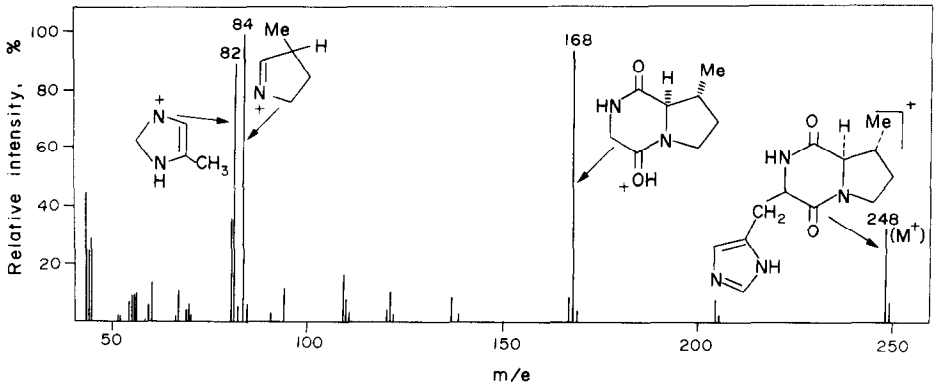


Fig. 3. Electron impact mass spectrum of the major metabolite of (3-Me-Pro)TRH produced by rat plasma.

tissues. However, chemical modification of TRH via the introduction of a *trans*-3-methyl substituent into the proline residue of the tripeptide conferred enhanced biological stability to the peptide. Methyl substitution also modified the pathway of metabolism. TRH was rapidly metabolized to histidine, confirming that both peptide bonds are rapidly hydrolysed. Histidine was only a minor metabolite in the case of the analogue, however, the major metabolite produced by plasma being the His-3-Me-Pro . NH_2 dipeptide observed as its corresponding diketopiperazine produced by spontaneous cyclization.

The means by which (3-Me-Pro)TRH shows greater resistance to metabolism is not completely clear but a steric protection of the C-terminal amide by the adjacent 3-methyl substituent is a possibility. It has been suggested [19, 20] that the mode of degradation of TRH involves an initial cleavage of the C-terminal amide to TRH-acid. Although not positively identified as an intermediate in these studies, this peptide may be short lived. Prasad and Peterkofsky [13] have shown that the TRH enzymatic activity of hamster hypothalamus extracts could be resolved into two components, one with C-terminal amidase activity and the other possessing pyroglutamyl peptidase activity. Results with (3-Me-Pro)TRH suggest that 3-methyl-substitution in proline inhibits the action of the C-terminal amidase. Such an action is evident in the production of the diketopiperazine His-(3-Me)-Pro; although His-3-Me-Pro . NH_2 readily cyclizes, authentic L-His-L-*trans*-3-methylproline (His-3-Me-Pro . OH) was found not to cyclize at physiological pH. Presumably this is because the carboxylate is ionized at pH 7.4 and is not susceptible to nucleophilic attack by the amino function in histidine. It follows, therefore, that in the degradation of (3-Me-Pro)TRH, His-3-Me-Pro . NH_2 and not His-3-Me-Pro . OH is the likely precursor to the observed diketopiperazine. The preferred formation of the latter from the analogue hormone could be evidence of a direct

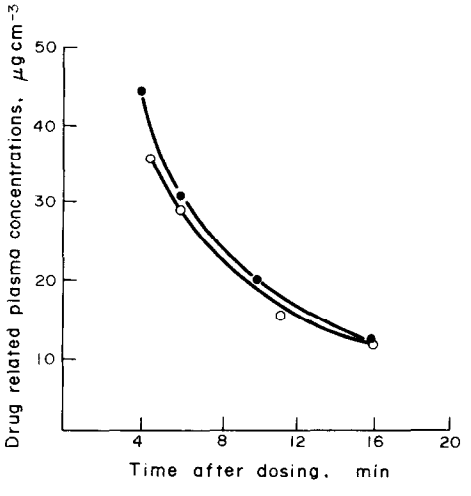


Fig. 4. Concentrations of total drug-related material in the plasma of rats following intravenous injection of [^{14}C]TRH or [^{14}C] (3-Me-Pro)TRH. All rats received the peptides at a dose of 10 mg/kg. Values for total radioactivity in plasma were converted to total drug-related material by means of the individual peptide specific activity. ●, TRH group; ○, (3-Me-Pro)TRH group.

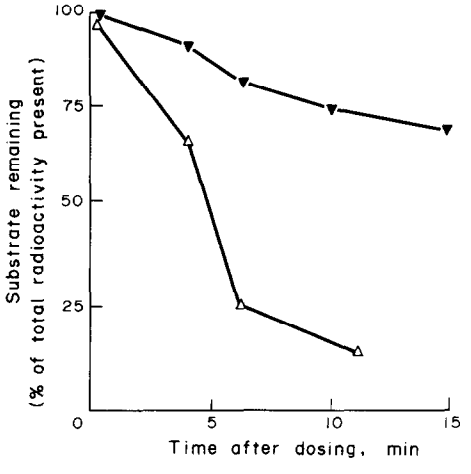


Fig. 5. Proportions of unchanged peptide in plasma after intravenous administration of [^{14}C]TRH or [^{14}C](3-Me-Pro)TRH to rats. All rats received the peptides at a dose of 10 mg/kg. ▼, (3-Me-Pro)TRH group; △, TRH group.

inhibition (by the 3-methyl group) of the action of the C-terminal amidase.

A translation of increased biological stability into greater *in vivo* bioavailability was illustrated in the case of the 3-methyl proline analogue of TRH. After intravenous administration to rats total drug-related material was cleared from the systemic circulation at similar rates for both peptides and it is therefore the stability of peptide present which primarily controls the absolute blood levels of unchanged drug. In a complementary fashion, the increased stability of the analogue to brain tissue would be expected to improve the relative central availability of the analogue assuming the efficiency of transport to the CNS is similar for both peptides. The increased stability of the analogue is therefore consistent with the improved CNS potency of the compound. The results described may be important in the design of peptide drugs with added protection against biological inactivation. Studies of this nature with other more potent analogues of TRH are presently under investigation in these laboratories.

Acknowledgements—We thank Mr. R. Martin for technical assistance and Dr. J. D. Bower for the samples of authentic diketopiperazines used in these studies.

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