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Inhibitors of Polyamine Biosynthesis. 3. (±)-5-Amino-2-hydrazino-2-methylpentanoic Acid, an Inhibitor of Ornithine Decarboxylase

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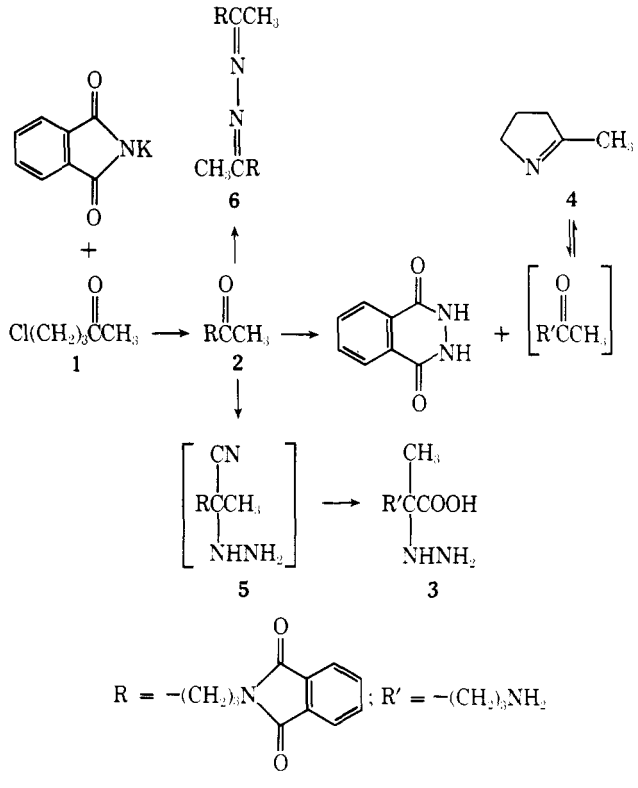
(±)-5-Amino-2-hydrazino-2-methylpentanoic acid [α-hydrazino-α-methyl-(±)-ornithine] was obtained from 1-phthalimidopentan-4-one by treatment with hydrazine and KCN followed by acid hydrolysis. The title compound was found in vitro to be a potent competitive inhibitor of ornithine decarboxylase obtained from the prostate glands of rats. This inhibition was abolished at high concentrations of pyridoxal phosphate. The title compound also blocked the increase in putrescine levels normally observed in bovine lymphocytes transformed by conconavalin A.

In an effort to elucidate the role of polyamines in proliferating tissues we have embarked on a program for the synthesis of specific inhibitors of the enzyme ornithine decarboxylase which is thought to be the rate-limiting step in the synthesis of the polyamines. In previous communications from this laboratory we have described the synthesis of α-alkyl- and α-aralkyl-(±)-ornithines and their evaluation as inhibitors of this enzyme.[1,2] The present report describes the synthesis of (±)-5-amino-2-hydrazino-2-methylpentanoic acid [α-hydrazino-α-methyl-(±)-ornithine] and its evaluation as an inhibitor of ornithine decarboxylase in vitro and in transforming lymphocytes. This ornithine analog was studied on the premise that since both α-methyl-(±)-ornithine and α-hydrazino-(±)-ornithine were found to be effective inhibitors of ornithine decarboxylase, the combination of these modifications might provide more potent inhibitor of this enzyme. Furthermore, α-methyl-α-hydrazino analogs of other amino acids were found to be potent inhibitors of their decarboxylases.[3]

Results and Discussion

The target compound was obtained using a modification of the Strecker synthesis as shown in Scheme I. 5-Chloro-2-pentanone (1) was treated with potassium phthalimide to provide **2** in moderate yields. Treatment of **2** with hydrazine and KCN at room temperature, followed by heating under reflux, provided phthalhydrazide in quantitative yields and a complex mixture of crude products which was treated in situ with concentrated hydrochloric acid. Purification of the product by ion-exchange resin provided the crude target compound **3** in 40% yield and a volatile compound which was identified as 2-methyl-Δ^1-pyrroline (**4**) in 50% yield. The separation of compound **4** was accomplished by treatment of the crude product after acid hydrolysis with Na_2CO_3 followed by steam distillation. The amine was separated from the distillate as the hydrochloride and picrate salts. The formation of compound **4** could be attributed to the facile cleavage of the phthalimide-protecting group prior to the formation of the hydrazinonitrile **5**. This was shown to be the case since mixing equimolar amounts of hydrazine and **2** at room temperature produced phthalhydrazide, unreacted **2**, and bis(1-methyl-4-phthalimidobutyl)hydrazone (**6**) in 40, 40, and 10% yields, respectively. The formation of **6** in the presence of limiting concentration of hydrazine dictated the use of an excess of hydrazine in subsequent reactions. Scheme I represents a proposed overall scheme for the formation of the isolated and identified compounds. In an attempt to improve the yields of **3**, compound **2** was initially treated with $NaHSO_3$ followed by treatment with KCN and hydrazine. This, however, did not increase the yield of **3**.

Scheme I



The 10,000g supernatant from extracts of prostate glands of rats was dialyzed for 12 hr and then used as the source of the enzyme ornithine decarboxylase for studies of the inhibition by **3**·HCl of the enzymatic decarboxylation of L-ornithine. The activity of ornithine decarboxylase was measured by determining the amount of $^{14}CO_2$ released from [^{14}C]carboxyl-(±)-ornithine in the presence of pyridoxal phosphate. Corrections were made for the nonenzymatic production of $^{14}CO_2$ by running controls which were identical with the experimental runs except that the tissue extracts were replaced with the homogenization solution. The activity of prostatic ornithine decarboxylase was found

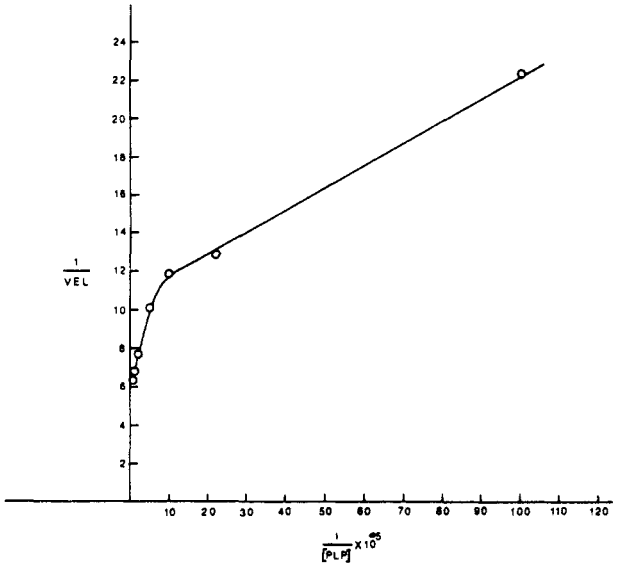


Figure 1. Effect of varying the concentration of pyridoxal 5′-phosphate on the rate of L-ornithine decarboxylation by ornithine decarboxylase from the prostate glands of rats. The concentration of L-ornithine used was 4.0×10^{-4} *M*.

to be strongly dependent on pyridoxal phosphate, and in the presence of a constant concentration of ornithine the velocity of ornithine decarboxylase at various concentrations of pyridoxal phosphate was not linear when graphed on a Lineweaver–Burk plot (Figure 1). Harik and Snyder have reported a similar finding with the ornithine decarboxylase from rat prostate glands.[4] Thus it was not feasible to use saturating concentrations of pyridoxal phosphate in our studies. Instead the highest concentration of pyridoxal phosphate used was 2×10^{-4} *M* which is well above physiological concentrations.

At a concentration of 10^{-5} *M* pyridoxal phosphate the ornithine decarboxylase from rat prostate glands has an apparent K_m for L-ornithine of 6×10^{-5} *M* and the V_{max} is 0.067 nmol of CO_2/mg of wet wt of tissue/hr. The addition of **3**·HCl at a concentration of 9.7×10^{-6} and 2.9×10^{-6} *M* repressed the production of $^{14}CO_2$. It appears from the Lineweaver–Burk plot of these data that **3**·HCl is a competitive inhibitor of decarboxylation of L-ornithine by ornithine decarboxylase with an apparent K_i of 3.0×10^{-6} *M*. Under these same test conditions the apparent K_i for α-methyl-(±)-ornithine hydrochloride is 2.0×10^{-5} *M*. Others have reported α-hydrazinoornithine to have an apparent K_i of 2.0×10^{-6} *M* for prostatic ornithine decarboxylase under similar reaction conditions.[4]

Table I. Effect of Pyridoxal Phosphate Concentration on the Rate of Decarboxylation of L-Ornithine Decarboxylase from Rat Prostate Glands in the Presence of Different Concentrations of **3**·HCl

Concn of pyridoxal phosphate, *M*	Concn of **3**·HCl, *M*	Vel (nmol of CO_2/mg of wet wt tissue/hr)
2×10^{-4}	1×10^{-3}	0.000
	1×10^{-4}	0.018
	1×10^{-5}	0.038
	0	0.042
1×10^{-4}	1×10^{-4}	0.003
	1×10^{-5}	0.037
	0	0.042
1×10^{-5}	1×10^{-5}	0.015
	3×10^{-6}	0.026
	0	0.040

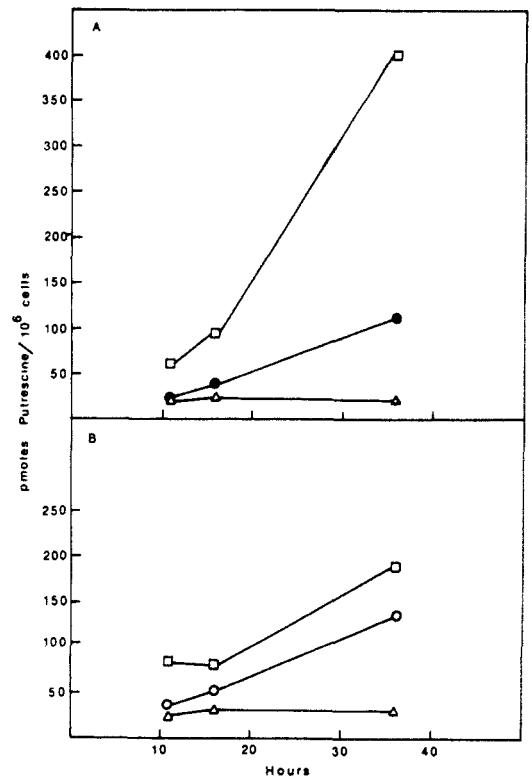


Figure 2. Effects of **3**·HCl on the increase of putrescine produced by exposing bovine lymphocytes to Con A. The data in A and B were determined from cells prepared on different days. Each point represents the mean of four determinations obtained from the analysis of two samples removed from each of duplicate flasks: control (△); Con A, 12 μg/ml (□); Con A and **3**·HCl, 1×10^{-3} (●); and Con A and **3**·HCl, 4×10^{-4} (○).

Harik and Snyder[4] have reported that the inhibition of ornithine decarboxylase by α-hydrazinoornithine was abolished in the presence of concentrations of pyridoxal phosphate which were 1.0×10^{-4} *M* or higher. On the other hand, the inhibition of ornithine decarboxylase by α-methyl-(±)-ornithine was only slightly affected by a 100-fold increase in pyridoxal phosphate concentration.[1] It was therefore of interest to examine the effect of increasing the concentration of pyridoxal phosphate on the inhibition of ornithine decarboxylase by **3**·HCl (Table I). The inhibition of ornithine decarboxylase by **3**·HCl was also highly dependent on the concentration of pyridoxal phosphate, and pyridoxal phosphate concentrations which were high relative to the concentration of **3**·HCl decreased the inhibition of ornithine decarboxylase. It is expected, therefore, that the nonlinear dependence of ornithine decarboxylase activity on the concentration of pyridoxal phosphate (Figure 1), together with the dependence of the inhibitory activity of **3**·HCl on the concentration of pyridoxal phosphate, will complicate the kinetics of the inhibition of the enzyme by **3**·HCl. The competitive pattern of inhibition observed from the Lineweaver–Burk plot, using only two concentrations of the inhibitor, may not necessarily represent the exact mechanism of inhibition at higher concentrations of the inhibitor.

Inhibition of ornithine decarboxylase in intact cells which are actively synthesizing polyamines would be expected to decrease the levels of putrescine in these cells. Such a decrease was produced by **3**·HCl in bovine lympho-

Table II. Effect of 3·HCl and α-Methyl-(±)-ornithine Monohydrochloride on Putrescine Levels in Transforming Bovine Lymphocytes

Treatment	Putrescine levels[a] (pmol/10^6 cells)
None	27.0 (24–30)
Con A (12 μg/ml)	58.5 (56–61)
Con A (12 μg/ml) and 3·HCl (4×10^{-4} *M*)	38.0
Con A (12 μg/ml) and α-methyl-(±)-ornithine (4×10^{-4} *M*)	32.5 (32–33)

[a]The values represent the mean (range) of putrescine levels in two aliquots of cell suspension removed from each of duplicate flasks 16 hr after the addition of Con A alone or with inhibitor.

cytes transformed by conconavalin A (Figure 2). Treatment of bovine lymphocytes with Con A increased the putrescine levels in these cells in a manner similar to that reported by others.[5] Addition of 3·HCl at a concentration of 10^{-3} *M* caused a complete block and at 4×10^{-4} *M*, an 80% block of the increase in putrescine levels normally observed 11 hr after treatment with Con A. At later times this inhibition was partly reversed and the levels of putrescine increased slightly. However, at all times tested, the levels of putrescine were much lower in transformed lymphocytes treated with 3·HCl than in untreated transformed lymphocytes. α-Methyl-(±)-ornithine also produced a block of the increase in putrescine levels in transformed lymphocytes and this effect of α-methyl-(±)-ornithine was slightly greater than that of 3·HCl (Table II). α-Hydrazinoornithine also was found to block net putrescine synthesis in rat hepatoma cells in culture and in the liver of intact rats.[9]

Conclusion

(±)-5-Amino-2-hydrazino-2-methylpentanoic acid (3·HCl) is a potent competitive inhibitor of ornithine decarboxylase obtained from the prostate glands of rats. It is approximately ten times more potent than α-methyl-(±)-ornithine and equipotent to α-hydrazino-L-ornithine in vitro. Surprisingly, 3·HCl was slightly less potent than α-methyl-(±)-ornithine in blocking the increase in putrescine levels in transformed bovine lymphocytes.

Experimental Section

The melting points were determined in open capillary tubes with a Thomas-Hoover apparatus and are uncorrected. Elemental analysis was performed by M-H-W Laboratories, Garden City, Mich., and by Galbraith Laboratories, Inc., Knoxville, Tenn. The ir spectra was obtained with a Perkin-Elmer 237B or Beckman IR-9 spectrophotometer. The NMR spectra were taken on a Varian A-60D in $CDCl_3$ or D_2O with Me_4Si or DSS, respectively, as internal standards. Mass spectra analyses were performed on AEI-MS30. All spectral data were consistent with the proposed structures. Radioactivity was measured using a Beckman LS-150 liquid scintillation counter.

(±)-5-Amino-2-hydrazino-2-methylpentanoic Acid (3·HCl). A suspension of 1-phthalimidopentan-4-one[1] (2, 36.0 g, 0.155 mol) in water (200 ml) was treated with hydrazine (16.0 ml, 0.47 mol) and the mixture was stirred for 0.5 hr at room temperature; at this time a clear solution was obtained. The solution was treated with KCN (10.6 g, 0.163 mol); the mixture was stirred for 0.5 hr at room temperature and then heated under reflux for 16–18 hr. The cooled and stirred solution was treated slowly with concentrated HCl (300 ml). The white precipitate formed was filtered and was identified as phthalhydrazide, 341–345° (lit.[6] 341–344°). The filtrate was concentrated under reduced pressure and the precipitate formed was filtered and identified as hydrazine dihydrochloride, mp 199–200° (lit.[6] 198–200°). The filtrate was treated with an equal volume of concentrated HCl and heated under reflux for 12–18 hr. The mixture was concentrated under reduced pressure and filtered and the filtrate was concentrated to a thick yellow oil. The residue was dissolved in H_2O (500 ml) and treated with Amberlite IR-120 ion-exchange resin (100 g) and the mixture was stirred for 2 hr. The resin was filtered, washed with successive portions of water until the washings were neutral, and transferred to a beaker containing 5 *N* NH_4OH (500 ml). The mixture was stirred for 0.5 hr and filtered and the filtrate was evaporated to a brown oil under reduced pressure. The residue was treated with 0.1 *N* HCl to a pH of 5 ± 0.25. The solution was concentrated to dryness to provide crude 3·HCl (12.8 g, yield 42%). The residue after three crystallizations (EtOH–H_2O) gave 3·HCl: mp 227–228°; ir (KBr) 3295, 2470, 2060, 1650, 1605, 1500, 1465, 1400, 1360, 800, 780, and 735 cm^{-1}; NMR (D_2O–DSS) δ 1.44 (s, 3 H), 1.79 (m, 4 H), 3.00 ppm (t, 2 H). Anal. ($C_6H_{15}N_3O_2$·HCl) C, H, N.

Isolation and Identification of 2-Methyl-Δ¹-pyrroline (4). The reaction of 2, hydrazine, and KCN followed by acid hydrolysis was carried out as described for the synthesis of 3·HCl. The crude residue obtained was not added to the ion-exchange resin but was treated with a solution of Na_2CO_3 to adjust the pH to 9.0. The mixture was distilled under vacuum and the distillate was divided into two portions. The first portion was treated with HCl and concentrated to dryness to provide 4·HCl which was a very hygroscopic solid. The second portion was extracted with ether. The ether extract was dried (Na_2SO_4) and treated slowly with a solution of picric acid in ether saturated with water and the solid obtained after three crystallizations (*n*-BuOH), mp 121–123° (lit.[7] 121–122.5°). The spectral characteristics of this product were consistent with literature values. In a separate run, 4·HCl was isolated in 52% yield.

Bis(1-methyl-4-phthalimidobutyl)hydrazone (6). A suspension of 2 (1.55 g, 0.005 mol) in water (15 ml) was treated with hydrazine (0.17 ml of 95% solution, 0.005 mol) and the mixture was stirred for 1 hr at room temperature but complete solution was not observed. The mixture was filtered and the precipitate (0.4052 g, 42%) was identified as unreacted 2. The filtrate was extracted with chloroform; the chloroform extract was dried (anhydrous Na_2SO_4) and evaporated to dryness. The residue (0.15 g, 13%) was recrystallized from $CHCl_3$–petroleum ether, mp 145–147°, and was identified as the title compound (6) by examination of its MS, NMR, and ir: NMR ($CDCl_3$–Me_4Si) δ 1.78 (s, 6 H), 1.98 (t, 4 H), 2.2 (m, 4 H), 3.7 (t, 4 H), and 7.63 ppm (m, 8 H); ir (KBr) 2960, 2930, 1770, 1710, 1635, 1390, and 715 cm^{-1}; MS *m/e* 298 (3), 270 (1.0), 229 (1.0), 198 (1.0), 160 (19.0), 147 (7.5), and 104 (18.5). Anal. ($C_{26}H_{26}N_4O_4$) C, H, N.

The original filtrate was cooled, stirred, and treated with concentrated HCl to provide phthalhydrazide (0.33 g, 41% yield).

Assay of Ornithine Decarboxylase Activity. The activity of ornithine decarboxylase was determined using the method described in ref 1. The procedure for the preparation and dialysis of the enzyme can also be found in ref 1.

Effects on Putrescine Levels in Transforming Lymphocytes. Lymphocytes were obtained from suprapharynegeal lymph nodes of cattle. The cells were purified as described by Fillingame and Morris[5] except they were applied to a column (15 × 5 cm i.d.) of glass beads (1-mm diameter) instead of a column of glass wool. The cells were cultured in the medium used by Fillingame and Morris.[5] Conconvalin A (12 μg/ml) was added to some culture flasks; others received various concentrations of inhibitors alone or in combination with Con A. Aliquots were removed from the flasks at appropriate times. The cells were harvested by centrifugation at room temperature for 5 min at 183g. The pellet was suspended in water and the solution was boiled for 20 min to destroy any endogenous *s*-adenosylmethionine. The amount of putrescine in this extract was then determined by the enzymatic procedure of Harik et al.[8]

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Synthesis and Biological Properties of the 2-L-β-(Pyrazolyl-1)alanine Analogs of Luteinizing Hormone-Releasing Hormone and Thyrotropin-Releasing Hormone

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The luteinizing hormone-releasing hormone (LH-RH) analog, <Glu-Pyr(1)Ala-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, and the thyrotropin-releasing hormone (TRH) analog, <Glu-Pyr(1)Ala-Pro-NH_2, were synthesized by azide couplings of the dipeptide hydrazide, <Glu-Pyr(1)Ala-$NHNH_2$, to the C-terminal octapeptide of LH-RH and to proline amide, respectively. In an ovariectomized, steroid-blocked rat assay, the LH-RH analog was found to have only 1% of the LH-releasing activity of the natural hormone. The TRH analog was 1.5 times more effective than TRH itself in releasing TSH in vivo from the anterior pituitary of mice. This peptide is one of two synthetic peptides so far discovered which are more potent than TRH.

β-(Pyrazolyl-1)alanine (A) and β-(pyrazolyl-3)alanine (B) are amino acids possessing a pyrazole ring system which is isomeric with, but in both cases considerably less basic[1] than, the imidazole nucleus of histidine (C). The β-(pyrazolyl-3)alanine analogs of a number of biologically active peptides,[2] including LH-RH[2] and TRH,[3,4] have been synthesized in order to investigate the importance of the histidine residues in maintaining activity. β-(Pyrazolyl-1)alanine has only been incorporated in the place of histidine in a RNase S-peptide analog[1] which was unable to produce an enzymatically active complex with the S-protein. We considered it worthwhile, therefore, to continue our studies on the function of histidine in LH-RH by preparing and assaying [L-β-(pyrazolyl-1)alanine[2]]-LH-RH and, at the same time, synthesizing [L-β-(pyrazolyl-1)alanine[2]]-TRH which, it appeared, could be made via a common starting material (see Figure 1).

Synthesis. Racemic β-(pyrazolyl-1)alanine was synthesized by the method of Reimlinger and Oth[5] and resolved into its L isomer by the method described by Sugimoto et al.[6] The L-amino acid methyl ester[1] was then used to prepare the dipeptide hydrazide, <Glu-Pyr(1)Ala-$NHNH_2$ (IV), which was coupled by the azide method to proline amide and to the C-terminal octapeptide (I)[2,7] of LH-RH to produce the tripeptide V and decapeptide VI, respectively.

The LH-RH analog was purified by partition chromatography on Sephadex G-25, followed by ion-exchange chromatography on CM-cellulose, and the TRH analog by chromatography on silica gel and on CM-cellulose.

Biological Results and Discussion. [Pyr(1)Ala[2]]-LH-RH was assayed (Table I) for LH-releasing properties at doses of 50 and 250 ng in ovariectomized rats pretreated with estrogen and progesterone, followed by radioimmunoassay[8] for serum LH. LH levels were compared with those obtained in control animals given saline and 1- and 5-ng amounts of LH-RH. The LH-RH analog was found to have only 1% of the activity of LH-RH itself, which is considerably lower than the 19% activity found previously[2] for the closely related [Pyr(3)Ala[2]]-LH-RH. The pyrazole groups of Pyr(1)Ala and Pyr(3)Ala and the imidazole ring of histidine have pK values[1] of 2.2, 2.5, and 6.0, respectively. Thus, reduction in the basicity of the side chain of the position two amino acid of the LH-RH decapeptide appears to lead to a concomitant lowering of gonadotropin-releasing activity. The side group of the Pyr(1)Ala-peptide is substituted via the pyrazole NH moiety and it is possible that its 1% of LH-RH activity is derived only from the aromatic character of the pyrazole ring, since [Phe[2]]-LH-RH[9] has virtually identical activity in this same assay system.

The TRH activity of [Pyr(1)Ala[2]]-TRH was determined by the procedure of Redding and Schally,[10] based on its ability to release TSH from the pituitary gland of mice pretreated with ^{125}I. The elevation of endogenous TSH increases the rate of release of labeled thyroid hormone from the thyroid gland, measured by the increase in blood radioactivity over that of control groups, and compared (Table II) to the response elicited by TRH. [Pyr(1)Ala[2]]-TRH was about 50% more active than TRH and thus about 30 times more active than [Pyr(3)Ala[2]]-TRH[3,4] which has only 5% of the activity of the parent hormone. Only one other analog, [(N^{im}-Me-His)[2]]-TRH,[11] has been found to be more potent than TRH. This peptide was about eight times more active than TRH and, like the Pyr(1)Ala peptide, contained a substitution on one of the nitrogen atoms of the aromatic ring system. In the same report,[11] it was demonstrated that a roughly linear relationship exists between decreasing basicity of the imidazole group in a varying environment in a number of analogs and increasing biological activity. This appears to be the reverse of the situation existing with LH-RH peptides and does not explain fully the surprisingly low activity of the less basic [Pyr(3)Ala[2]]-TRH.

Experimental Section

Asymmetric amino acids were of the L configuration. Melting points were uncorrected. Microanalyses were carried out by PCR, Inc., Gainesville, Fla. Amino acid analyses were performed on a Beckman Model 119 amino acid analyzer equipped with a System AA computing integrator using the single-column method. Peptides were hydrolyzed (110°, 18 hr) in vacuo in 4 *M* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole.[12] Pyr(1)Ala was eluted in the same position as serine and, under the normal hydrolysis conditions using either methanesulfonic acid or 6 *M* HCl, was approximately 30% destroyed. The Pyr(1)Ala-LH-RH and TRH peptides were, therefore, hydrolyzed for 18, 36, and 72 hr and nanomoles of Pyr(1)Ala were calculated by extrapolation to zero time. R_f^1, R_f^2, R_f^3, R_f^4, R_f^5, and R_f^6 values refer to TLC on silica gel using the solvent systems $CHCl_3$–MeOH (25:1), 1-BuOH-