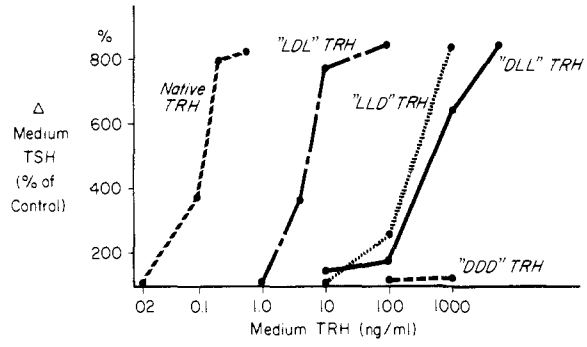

Figure 1.

medium to permit TSH release to equilibrate at a basal level. Six tissues were used per test substance. The medium was then removed and discarded and replaced by 1 ml of KRB for the succeeding 1-hr control period. Medium was then replaced again with media containing TRH or analogs at concentrations indicated in Figure 1 (abscissa). Medium TSH concentrations during control and experimental periods were determined by a specific radioimmunoassay for rat TSH.[11] Incremental changes in medium TSH are expressed as per cent of control [TSH (hr 2)/TSH (hr 1) × 100].

Native TRH yielded a sigmoidal dose-response function between 0.020 and 0.2 ml (Figure 1). LDL-TRH gave a parallel dose-response funct shifted to the right. The observed potency of LDL-TRH was about 2-3% of native TRH activity. In contrast, the analogs LLD- and DLL-TRH possessed only about 0.1% of native TRH activity. DDD-TRH failed to exhibit any TSH-releasing activity with medium concentrations as high as 1 μg/ml.

In order to test for inhibitory properties, hemipituitaries were incubated with a submaximal TRH stimulus (100 pg/ml) and substimulating concentrations of the four analogs.

The LDL analog did not inhibit thyrotropin release at a concentration 20 times that of the hormone nor did the LLD, DLL, or DDD isomers at concentrations 1000 times that of TRH.

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Effect of Simple Amino Acid Replacements on the Biological Activity of Luteinizing Hormone-Releasing Hormone

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Six decapeptides having structures based on the LH-RH sequence, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg- Pro-Gly-NH_2, have been synthesized by modifications of the solid-phase technique *via* protected peptide intermediates. Thus, pyroglutamic acid, tryptophan, serine, glycine, proline, and glycine amide residues in the LH-RH sequence were replaced by leucine, tyrosine, threonine, isoleucine, alanine, and glycine dimethylamide, respectively. The compounds were assayed *in vivo* and found to possess the following LH-releasing potencies relative to pure, natural LH-RH: [Leu[1]]-LH-RH, 0.003%; [Tyr[3]]-LH-RH, 0.13%; [Thr[4]]-LH-RH, 19%; [Ile[6]]-LH-RH, 0.034%; [Ala[9]]-LH-RH, 0.8%; and [(Gly-dimethylamide)[10]]-LH-RH, 14%.

Recent studies[1,2] on the effects of replacement of tyrosine in position 5 of LH-RH by closely related amino acids with aromatic side chains established that the phenolic hydroxyl group does not contribute significantly toward biological activity. This paper reports methods of synthesis and biological characteristics of decapeptides containing replacements for many of the other residues.

Of the amino acids with functional side chains in LH-RH, cyclic pyroglutamic acid was replaced by leucine, tryptophan by tyrosine, and serine by the closely related threonine. While this work was in progress, data on the threonyl peptide, synthesized by fragment condensation, were reported by Fujino, *et al.*[3] The glycine residue in the sterically crowded center of the molecule and the conformationally important proline at position 9 were particularly intriguing and were replaced by isoleucine and alanine, respectively. Finally, the importance of the amide part of C-terminal glycine was examined by replacing it with a dimethylamide group.

Synthesis. All protected peptides were prepared on a resin support by versions of the Merrifield solid-phase method[4] which have been described in part previously.[1,2,5] Protected peptide amides were cleaved from the resin by treatment with ammonia in methanol or, in the case of [(glycine-dimethylamide)[10]]-peptide, with dimethylamine.

In all the peptides the following groups were used for the protection of sensitive side chains during synthesis: arginine, N^G-tosyl; serine, threonine; and tyrosine, *O*-ben-

Table I. LH-RH Activity of Various Analogs Compared with That of the Natural LH-RH in Ovariectomized, Estrogen–Progesterone Treated Rats

Sample	Dose, ng/rat	Mean LH, ng/ml + S.E.	Potency, %[a] (95% confidence limits)
Saline		5.1 ± 0.4	
Natural LH-RH	0.5	10.9 ± 1.4	
	2.5	27.9 ± 3.7	
[Leu^1]-LH-RH	5000	6.4 ± 0.8	0.003 (0.0012–0.0061)
	25000	14.8 ± 0.6	
Saline		6.8 ± 0.7	
Natural LH-RH	0.5	14.1 ± 2.3	
	2.5	38.0 ± 5.9	
[Tyr^3]-LH-RH	1000	25.9 ± 4.3	0.013 (0.074–0.26)
	5000	58.3 ± 1.7	
Saline		4.02 ± 0.2	
Natural LH-RH	0.5	6.4 ± 0.5	
	2.5	16.9 ± 2.9	
[Ile^6]-LH-RH	1000	5.7 ± 0.5	0.034 (0.012–0.077)
	5000	13.2 ± 2.4	
Saline		7.2 ± 1.4	
Natural LH-RH	0.5	18.4 ± 1.5	
	2.5	52.4 ± 6.5	
[Thr^4]-LH-RH	3	16.6 ± 2.1	19 (12–30)
	15	61.2 ± 7.5	
Saline		4.5 ± 1.2	
Natural LH-RH	0.5	13.9 ± 0.5	
	2.5	61.3 ± 6.2	
[Ala^9]-LH-RH	50	24.3 ± 2.9	0.85 (0.2–3.0)
	250	44.6 ± 17.6	
Saline		5.1 ± 0.7	
Natural LH-RH	0.5	13.9 ± 1.1	
	2.5	40.3 ± 4.4	
[(Gly-NMe$_2$)^{10}]-LH-RH	2	8.1 ± 2.2	14 (7–23)
	10	28.6 ± 3.9	

[a] Pure natural LH-RH[14] accepted as 100%.

zyl. Histidine was incorporated into the threonyl peptide as its N^{im}-benzyl derivative, requiring the use of sodium in liquid ammonia reduction[6] for final removal of protecting groups. Previously,[2,7] no evidence has been found by us to indicate the presence of D-histidine in final peptides prepared by coupling with this derivative. However, reports[8,9] indicating that racemization can occur led us to use N^{im}-tosyl protection for histidine in all but one of the remaining peptides. This group is removed[2] completely during the ammonolysis step and the remaining protecting groups were then removed by treatment[10] with hydrogen fluoride in the presence of anisole. Trial treatments of Boc-His(Tos) with 1 *M* HCl in acetic acid revealed that the tosyl group was partially removed under these conditions, an event that could have deleterious effects during later couplings. Boc-His(DNP)[11] was used with improved results in the synthesis of [Leu^1]-LH-RH. The DNP group appears to be stable to the acid reagent and yet is rapidly and completely removed[12] during ammonolytic cleavage of peptides from the resin.

In initial syntheses of LH-RH itself, it was noticed that roughly 10% contamination of the protected peptide resulted by a component possessing a much higher R_f value than the major product. This compound was isolated by preparative tlc and shown to be the C-terminal Boc-octapeptide resulting from incomplete removal of the Boc group protecting Trp. This was not rectified even when a 1% resin support was used. Similarly, the protected peptides prepared here were also contaminated to various extents with fast-moving components visualized as Ehrlich positive, Pauly negative spots which also probably corresponded to the C-terminal Boc-octapeptides. Fortunately, the increased solubility of these enabled them to be removed by careful reprecipitation of the mixtures. In an effort to overcome this problem, TFA was substituted for the HCl reagent during deblocking of tryptophan. However, although an improvement, this was still not completely effective. For the [Leu^1]-peptide, HCl in formic acid, a more powerful reagent[13] for removing Boc groups, proved to be more efficient, the resulting 1-formyl group on Trp being removed during ammonolysis from the resin.

Hydrogen fluoride deprotected peptides were subjected to an initial purification step involving gel filtration on Sephadex G-25 in 0.2 *M* AcOH. This process removed incompletely deprotected peptide and also peptide-containing benzylated aromatic residues resulting from Friedel–Crafts acylation,[14] all of which are considerably retarded in relation to the main product. Peptides were finally purified with an exponential gradient of NH_4Ac buffers on CM cellulose.

Biological Results. LH-RH activities (Table I) were determined *in vivo* by the stimulation of LH release at two dose levels in ovariectomized rats pretreated with estrogen and progesterone,[15,16] followed by radioimmunoassay[17] for LH. Serum LH levels were compared with those obtained after administration of saline and two doses of natural LH-RH.

The biological potency of the decapeptide has been shown to be extremely sensitive to changes in the N-terminal position. Thus, elimination[18] of <Glu and also its replacement by proline[3,19] result in virtually complete loss of activity. It is interesting to note, however, that [Glu^1]-LH-RH[18] still retains 5.7% activity despite the linear structure of glutamic acid. The [Leu^1]-peptide has very little activity, suggesting that the side-chain carbonyl group in this position is of great importance.

It has been reported[18,20] that replacement of Trp in position 3 by Phe results in retention of 0.43% activity.

Therefore, the low potency of [Tyr^3]-LH-RH (0.13%) was predictable. The even lower activity may be due to the presence of the phenolic hydroxyl group close to a position usually occupied by the more basic indole ring.

The significant activity of [Ala^4]-LH-RH (8.6%) synthesized by Geiger, *et al.*,[21] demonstrated that the hydroxyl group of serine is not absolutely essential for biological activity. It was expected that the [Thr^4]-peptide would retain considerable potency. The figure of 19% is higher than that reported[3] previously. The replacement of glycine in position 6 of an already crowded structure by a bulkier amino acid would be expected to severely alter the stereochemistry of the molecule and, hence, biological potency. The [Ile^6] compound is almost completely inactive (0.034%). Replacement of proline by alanine also results in severe loss of activity (0.8%), again presumably due to conformational disruption. It has been shown[22] that the C-terminal glycine amide portion of LH-RH is not essential for existence of high levels of hormonal activity. This is confirmed by the 14% potency of [(glycine-dimethylamide)^{10}]-LH-RH.

Experimental Section

Amino acid derivatives used as starting materials were, with the exception of glycine, the pure L isomers purchased from Bachem, Inc., Marina del Rey, Calif. Microchemical analyses were performed in duplicate by Galbraith Laboratories, Inc., Knoxville, Tenn., on samples which were dried *in vacuo* over P_2O_5 at 80°. Amino acid analyses were carried out on samples which were hydrolyzed (18 hr) in 6 *M* HCl containing 4% thioglycolic acid.[23] All operations on the resin took place in a Beckman Model 990 automatic peptide synthesizer. The following tlc systems were employed: R_f^1, *n*-BuOH–AcOH–H_2O (4:1:5, upper phase); R_f^2, *n*-BuOH–AcOH–Pyr–H_2O (15:3:10:12); R_f^3, *n*-BuOH–EtOAc–AcOH–H_2O (1:1:1:1); R_f^4, EtOAc–Pyr–AcOH–H_2O (5:5:1:3); R_f^5, EtOH–H_2O (7:3). Sample sizes of *ca.* 30 μg were spotted on Brinkmann precoated layers and solvent fronts were allowed to travel *ca.* 15 cm.

Boc-protected amino acids (3.0 mmol) were coupled successively in the presence of DCI (3.0 mmol) to a 2% cross-linked, polystyrene–divinylbenzene–glycine resin (1.0 mmol of Gly) by a procedure which has been described previously.[2,5] Boc-protecting groups were eliminated at each stage by treatment with 1 *M* HCl in glacial AcOH, 1% mercaptoethanol being included in this reagent after incorporation of Trp. TFA containing 1% mercaptoethanol was used for more efficient removal of the relatively unreactive Boc group on Trp. In the synthesis of [Leu^1]-LH-RH, 0.5 *M* HCl in anhydrous formic acid was completely effective in deblocking Trp and appeared to be the reagent of choice. Boc-Arg(Tos), Boc-His(Bzl), and <Glu were coupled in a 1:3 mixture of DMF–CH_2Cl_2 for increased solubility and the remaining amino acids in CH_2Cl_2.

HF-cleaved peptides were first subjected to elution on a column (2.7 × 61 cm) of Sephadex G-25 in 0.2 *M* AcOH and always emerged as a major peak followed by smaller, broader peaks of varying intensity corresponding to partially protected peptide and peptide-containing benzylated tyrosine. All peptides were finally applied on a column (1.4 × 94 cm) of CM-cellulose equilibrated with 0.002 *M* NH_4Ac buffer at pH 4.6. A pH and concentration gradient was begun immediately by introducing 0.1 *M* NH_4Ac (pH 7.0) through a 250-ml flask containing starting buffer. Peptides in the effluents were located by measurement of OD at 280 nm. All but one of the peptides emerged in the region between 950 and 1200 ml. The more basic [Leu^1]-LH-RH was eluted between 600 and 700 ml by using 0.2 *M* NH_4Ac (pH 7.0) as the second buffer.

<Glu-His(Bzl)-Trp-Thr(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH_2 (I). Amino acids were joined to the glycine-resin (2.94 g, 1.00 mmol) to yield the protected peptide-resin (4.40 g, 94%). This material (1.5 g) was suspended in dry MeOH (90 ml) which was then saturated at 0° with anhydrous NH_3 and allowed to stir (18 hr) at room temperature in a tightly stoppered flask. Ammonia was partially removed at the water pump, the mixture filtered, and the resin extracted with DMF (three 15-ml portions). Methanol was removed from the filtrates *in vacuo* and the protected peptide precipitated by the addition of a large excess of EtOAc. The white powder (419 mg) was purified by reprecipitation from boiling methanol (186 mg, 33% based on initial Gly content of resin): single spot to Ehrlich reagent and I_2 vapor; R_f^1 (silica), 0.47. Amino acid analysis gave Trp, 0.95; NH_3, 0.96; Arg, 1.00; Thr, 0.86; Glu, 0.99; Pro, 0.84; Gly, 2.20; Leu, 1.06; Tyr, 0.92. *Anal.* ($C_{84}H_{102}N_{17}O_{15}S\cdot 3H_2O$) C, H, N.

<Glu-His-Trp-Thr-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (II). The protected peptide I (100 mg) was treated with Na in dry, redistilled NH_3 under the usual conditions.[1] The reduced peptide was first desalted by elution on Sephadex G-15 in 50% AcOH and then chromatographed on CMC to yield a white powder (37 mg, 50%): $[\alpha]^{24}D$ −50° (*c* 0.96, 0.1 *M* AcOH) [lit.[3] $[\alpha]^{23}D$ −56.6° (5% AcOH)]; single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f^1 (cellulose), 0.56; R_f^3 (silica), 0.59; R_f^5 (silica), 0.31; single component moving in the direction of the cathode after tle at pH 4.6 and 6.4 in pyridine acetate buffers. Amino acid analysis gave Trp, 1.01; His, 0.95; NH_3, 1.12; Arg, 1.00; Thr, 0.91; Gly, 0.98; Pro, 1.05; Gly, 2.02; Leu, 1.00; Tyr, 0.90.

<Glu-His-Tyr(Bzl)-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH_2 (III). Successive couplings to the glycine resin (2.94 g, 1.00 mmol) gave the protected peptide resin (4.92 g, 125%). Ammonolysis of part of this (2.35 g) yielded the protected peptide (835 mg) which was allowed to precipitate from a minimum volume of boiling ethanol to give a white powder (453 mg, 58%): single spot to Pauly reagent and I_2 vapor; R_f^1 (silica), 0.30. Amino acid analysis gave His, 1.09; NH_3, 0.91; Arg, 0.93; Ser, 0.96; Glu, 0.95; Pro, 1.02; Gly, 1.97; Leu, 1.00; Tyr, 1.61. *Anal.* ($C_{81}H_{100}N_{16}O_{16}S\cdot 3H_2O$) C, H, N.

<Glu-His-Tyr-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (IV). The protected peptide III (150 mg), anisole (3.5 ml), and HF (20 ml) were stirred at 0° for 1 hr. After removal of the HF *in vacuo* the sticky peptide was extracted into 0.1 *M* AcOH (50 ml) and the solution washed with EtOAc (four 10-ml portions). Lyophilization of the aqueous layer gave the crude peptide which was chromatographed to yield peptide IV (42 mg, 36%): $[\alpha]^{25}D$ −53° (*c* 0.69, 0.1 *M* AcOH); single spot to Pauly reagent and I_2 vapor; R_f^1 (cellulose), 0.37; R_f^2 (cellulose), 0.66; R_f^4 (silica), 0.56. Amino acid analysis gave His, 1.00; NH_3, 1.23; Arg, 0.96; Ser, 1.06; Glu, 0.98; Pro, 1.00; Gly, 2.04; Leu, 1.00; Tyr, 2.09.

<Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Ile-Leu-Arg(Tos)-Pro-Gly-NH_2 (V). Successive couplings to the glycine-resin (2.94 g, 1.00 mmol) gave the protected peptide-resin (4.63 g, 113%). Ammonolysis of part of this (2.50 g) yielded the protected peptide (699 mg) which was precipitated from boiling MeOH by the addition of EtOAc (235 mg, 27%): single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f^1 (silica), 0.32. Amino acid analysis gave Trp, 0.83; His, 0.89; NH_3, 1.50; Arg, 1.10; Ser, 0.73; Glu, 0.92; Pro, 1.15; Gly, 1.00; Ile, 1.04; Leu, 1.04; Tyr, 1.00. *Anal.* ($C_{80}H_{100}N_{17}O_{15}S\cdot 4H_2O$).

<Glu-His-Trp-Ser-Tyr-Ile-Leu-Arg-Pro-Gly-NH_2 (VI). The protected peptide V (200 mg), anisole (4.5 ml), and HF (25 ml) were stirred at 0° (1 hr). Extraction of the peptide followed by chromatography gave the required material (33 mg, 20%): $[\alpha]^{25}D$ −57° (*c* 0.76, 0.1 *M* AcOH); single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f^1 (cellulose), 0.47; R_f^2 (cellulose), 0.81; R_f^3 (silica), 0.59. Amino acid analysis gave Trp, 0.84; His, 1.06; NH_3, 0.97; Arg, 1.00; Ser, 0.95; Glu, 1.09; Pro, 1.00; Gly, 1.07; Ile, 0.91; Leu, 0.97; Tyr, 0.97.

<Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Ala-Gly-NH_2 (VII). Successive couplings to the glycine-resin (2.94 g, 1.00 mmol) gave the protected peptide-resin (4.43 g, 105%). Ammonolysis of the resin (2.40 g) yielded crude, protected peptide (766 mg) which was allowed to precipitate from a minimum volume of MeOH (268 mg, 32%): single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f^1 (silica), 0.24. Amino acid analysis gave Trp, 0.87; His, 0.93; NH_3, 1.48; Arg, 1.01; Ser, 0.75; Glu, 0.94; Pro, 0.98; Gly, 1.00; Ala, 1.00; Leu, 1.03; Tyr, 0.91. *Anal.* ($C_{74}H_{91}N_{17}O_{15}S\cdot 3H_2O$) C, H, N.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Ala-Gly-NH_2 (VIII). Peptide VII (150 mg) upon deprotection with HF and column chomatography gave [Ala^9]-LH-RH (20 mg, 18%): $[\alpha]^{25}D$ −30° (*c* 1.09, 0.1 *M* AcOH); single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f^1 (cellulose), 0.51; R_f^2 (cellulose), 0.71; R_f^3 (silica), 0.53. Amino acid analysis gave Trp, 0.94; His, 1.06; NH_3, 1.31; Arg, 1.03; Ser, 0.72; Glu, 1.10; Gly, 0.94; Ala, 0.97; Leu, 0.97; Tyr, 1.03.

<Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NMe_2 (IX). Successive couplings to the glycine-resin (2.94 g,

1.00 mmol) gave the protected peptide-resin (4.49 g, 107%). Part (2.04 g) of this was stirred at 0° in the presence of dimethylamine (20 ml) for 3 hr. After this time, excess amine was slowly allowed to evaporate at room temperature. The protected peptide was extracted in the usual fashion to yield 430 mg of yellow powder. This was purified by precipitation from MeOH with EtOAc (200 mg, 27%): single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f^1 (silica), 0.30. Amino acid analysis gave Trp, 0.80; His, 0.90; Arg. 1.01; Ser, 0.80; Glu, 0.95; Pro, 1.04; Gly, 1.02; Leu, 1.00; Tyr, 0.96. *Anal.* ($C_{78}H_{97}N_{17}O_{15}S\cdot 4H_2O$) C, H, N.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NMe$_2$ (X). Peptide IX (150 mg) upon deblocking with HF followed by column chromatography gave the required peptide X (36 mg, 32%): $[\alpha]^{25}D$ −53° (c 0.93, 0.1 *M* AcOH); single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f^1 (cellulose), 0.40; R_f^2 (cellulose), 0.78; R_f^4 (silica), 0.57. Amino acid analysis gave Trp, 0.85; His, 1.00; Arg, 0.97; Ser, 0.73; Glu, 0.90; Pro, 1.00; Gly, 2.07; Leu, 1.08; Tyr, 1.00.

Leu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH$_2$ (XI). Successive couplings of amino acids [including Boc-His(DNP)] to the glycine-resin (2.94 g, 1.00 mmol) gave the mustard-colored, protected peptide-resin (4.85 g, 115%). Ammonolysis of part (2.40 g) of this in MeOH (120 ml) rapidly produced a deep yellow solution which was stirred at room temperature (18 hr) in a tightly stoppered flask. Excess NH_3 was partially removed at the water pump, the mixture filtered, and the resin extracted with DMF until the filtrate was colorless. Methanol was removed *in vacuo* from the combined filtrates and the protected peptide precipitated from the bright yellow DMF solution with a large excess of EtOAc. Upon filtration the filter cake was washed extensively with EtOAc to remove contaminating 2,4-dinitroaniline. Drying gave a white powder (770 mg, 98%), a sample of which was precipitated from MeOH to give a single spot to Ehrlich and Pauly reagents and I_2 vapor, R_f^2 (silica), 0.31. Amino acid analysis gave Trp, 0.84; His, 0.97; NH_3, 1.20; Arg, 1.00; Ser, 0.71; Pro, 0.97; Gly, 2.00; Leu, 2.18; Tyr, 0.83. *Anal.* ($C_{77}H_{99}N_{17}O_{14}S\cdot 4H_2O$) C, H, N.

Leu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$ (XII). The protected peptide XI (150 mg) was deprotected with HF and purified by column chromatography (86 mg, 77%): $[\alpha]^{25}D$ −34° (c 0.95, 0.1 *M* AcOH); single spot to Ehrlich and Pauly reagents, I_2 vapor, and ninhydrin; R_f^1 (cellulose), 0.52; R_f^2 (cellulose), 0.74; R_f^3 (silica), 0.54. Amino acid analysis gave Trp, 1.10; His, 1.00; NH_3, 0.95; Arg, 1.00; Ser, 0.74; Pro, 0.96; Gly, 1.88; Leu, 2.12; Tyr, 0.96.

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