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Synthesis and Biological Evaluation of LH and FSH Releasing Hormone and Its Analogs†

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Details and additional information about another synthesis of LH-RH/FSH-RH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I), are described. In order to obtain enough highly purified material, the synthesis was performed by stepwise elongation according to the conventional method for peptide synthesis. The product was purified effectively by column chromatography on CM-Sephadex C-25. Homogeneity of the purified product was confirmed by tlc as well as by elementary analysis and acid and enzyme hydrolyses. The *in vivo* and *in vitro* activities of the synthetic LH-RH/FSH-RH (I) were of the same order of magnitude as those of pure natural LH-RH/FSH-RH. On the other hand, nonapeptide amide, des-pGlu^1-LH-RH/FSH-RH (II), and octapeptide amide, des-pGlu^1-des-His^2-LH-RH/FSH-RH (III), were, at best, only very weakly active. In addition, I stimulated cyclic AMP formation in rat anterior pituitary, while II and III exhibited no stimulation.

The decapeptide amide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 was prepared by stepwise elongation according to the conventional method for peptide synthesis. The product was purified by column chromatography on CM-Sephadex C-25. Homogeneity of the product was confirmed by tlc as well as elementary analysis and acid and enzymic hydrolyses. Because of lack of the α-amino group at the N terminus of the decapeptide amide, stereohomogeneity of the synthetic peptide was assessed by aminopeptidase-M (AP-M) digestion of nonapeptide amide H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (des-pGlu^1-LH-RH/FSH-RH). The *in vivo* and *in vitro* LH-RH activities of the synthetic decapeptide amide were of the same order of magnitude as those of pure natural porcine LH-RH/FSH-RH. The *in vitro* FSH-RH activity was also compared.

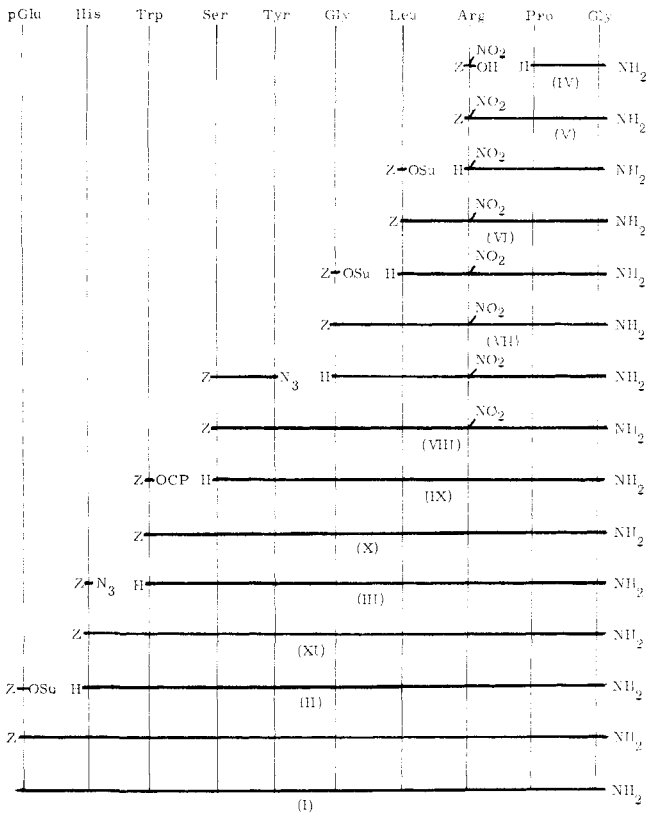
Studies by Schally and his group[2-5] led to isolation of porcine LH and FSH releasing hormone (LH-RH/FSH-RH) and determination of the structure as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I). This structure was confirmed by the synthesis of the decapeptide amide possessing identical properties with those of LH-RH/FSH-RH isolated from porcine hypothalami.[1,6-12] This communication provides details and additional information about another synthesis of the decapeptide amide.

In order to obtain enough material for further investigation of this hormone, we performed the synthesis according to the conventional method for peptide synthesis. The well-planned synthesis excluded any possibility of the presence of by-products in our LH-RH/FSH-RH preparation. LH-RH and FSH-RH activities of our synthetic decapeptide amide (I) were shown to be identical with those of pure natural porcine LH-RH/FSH-RH, while both nonapeptide amide, H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Des-pGlu^1-LH-RH/FSH-RH) (II), and octapeptide amide, H-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Des-pGlu^1-des-His^2-LH-RH/FSH-RH) (III), were, at best, only very weakly active. The *in vivo* LH-RH activity of II and III was reported by Schally, *et al.*[13]

Synthesis. Synthesis of I was carried out according to the route shown in Scheme I. H-Pro-Gly-NH_2·HBr (IV) was used as the starting material. The mixed anhydride of Z-Arg(NO_2)-OH[14] was coupled with IV to yield V. Decarbobenzoxylation of V with 25% HBr in AcOH followed by coupling with Z-Leu-OSu[15] produced VI, which was decarbobenzoxylated by the same treatment. The resulting partially deblocked material was coupled with Z-Gly-OSu[15] to give protected pentapeptide amide VII. This material was partially deblocked in the same manner as above and then combined with Z-Ser-Tyr-N_3, derived from the corresponding hydrazide,[16] to yield VIII. It has been known that the treatment of a Ser-containing peptide with HBr-AcOH leads to O-acetylation of the Ser residue.[17,18] In addition, this treatment might cause partial destruction of Trp residue. Ac-

†For the preliminary communication, see ref 1. The amino acid residues except glycine are of the L configuration.

Scheme I



cordingly, we selected catalytic hydrogenation for decarbobenzoxylation of protected peptides containing Trp and/or Ser residues. In the case of VIII, hydrogenolysis resulted advantageously in the formation of H-Ser-Tyr-Gly-Leu-Arg(H^+)-Pro-Gly-NH_2 (IX) in the form of acetate by simultaneous removal of Z and NO_2 functions. Interaction of IX with Z-Trp-OTCP[19] gave X, which was hydrogenated. The azide coupling of the resulting product, des-pGlu[1]-des-His[2]-LH-RH/FSH-RH (III), with Z-His-N_2H_3[20] produced XI, which was purified by column chromatography on CM-Sephadex C-25. Hydrogenolysis of the purified XI yielded nonapeptide amide, des-pGlu[1]-LH-RH/FSH-RH (II). Coupling of II with Z-pGlu-OSu followed by hydrogenation produced a crude I and the product was purified by chromatography on CM-Sephadex C-25 column.

Isolation and Purification. Isolation of each of intermediates V-VIII and X from the reaction mixtures was accomplished effectively by the countercurrent distribution between 1-BuOH and H_2O in separatory funnels. The product thus obtained from 1-BuOH layers was purified by precipitation from MeOH with AcOEt.

Purification of decapeptide amide I and Z-nonapeptide amide XI was conducted by chromatography on CM-Sephadex C-25 column using NH_4OAc buffer (pH 6.5) as an eluent (Figure 1). Location of the products was detected by OD measurement at 278 mμ and tlc on silica gel. Decapeptide amide I was eluted with 0.1 and 0.125 *M* NH_4OAc buffer. A small amount of the unreacted II, which was present as contaminant in the crude preparation of I, could readily be removed by this procedure. The more basic unreacted II required higher concentration of buffer (0.3 *M* NH_4OAc) for elution. Finally, the product dissolved in 1 *M* AcOH was desalted by gel filtration on Bio-Gel P-2.

For assessment of stereohomogeneity, II was subjected to AP-M digestion,[21] since the final product I lacks a free amino group at the N terminus. Amino acid ratios in the digest

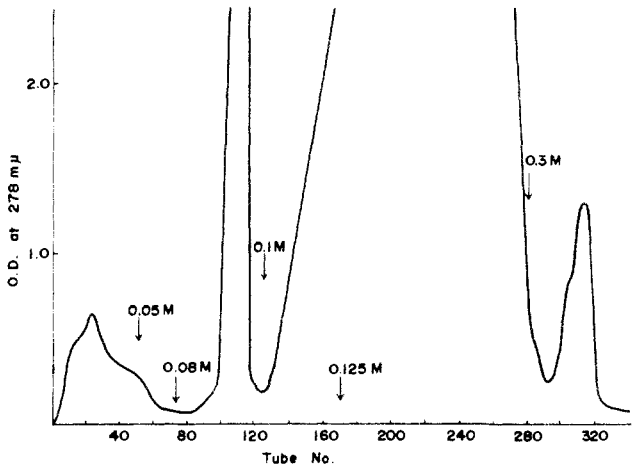


Figure 1. Stepwise gradient chromatography of crude preparation of I (1.8 g) on a column (3 × 10 cm) of CM-Sephadex C-25 using NH_4OAc buffer (pH 6.5) as an eluent. Fractions of 17 g were collected. The desired pure product I was located in fractions of tube number 131-276.

were those predicted by theory with the exception of Arg and Pro. These two amino acids were not detected in the digest and similar results were observed in AP-M digestion of a peptide possessing imide linkage such as His-Pro[22] or Asn-Pro[23] in the molecule.

The highly purified I, II, and III behaved as a single component respectively on tlc in the two solvent systems and their acid hydrolysates contained the constituent amino acids in the theoretical ratios. Since Trp was decomposed by acid hydrolysis under the condition used in this experiment, its content in synthetic I was estimated by the method of Bencze and Schmid.[24]

Hormonal Activities. The LH-RH activity of I, II, and III was determined *in vivo* by stimulation of release of LH in ovariectomized rats pretreated with estrogen and progesterone.[2,3,25] LH levels were measured by radioimmunoassay according to the method described by Niswender, *et al.*[26] The results are expressed in terms of NIH-LH-S-17. Responses to synthetic peptides were examined at two dose levels. Serum LH levels after injecting a sample were compared with those observed on administration of saline and of two doses of pure natural LH-RH/FSH-RH. The data are given in Table I. The results indicate that the three synthetic LH-RH/FSH-RH preparations possess the same or nearly same potency as that of pure natural one. Slight discrepancy, though not significant statistically, of the potency among the preparations seems to be due to difference in peptide content of the lyophilized samples, which were dried *in vacuo* at room temperature for 24 hr. According to amino acid analysis of the samples before bioassay, the peptide contents in the three preparations were 60, 65, and 78%, respectively. The peptide content in pure natural LH-RH/FSH-RH preparation used for the comparison was 67.5% and the hormonal activity was expressed on the basis of the corrected weight. Therefore, the actual corrected potency of our synthetic LH-RH/FSH-RH preparations fits perfectly in the correct range. Immediately after drying *in vacuo* at 50° for 48 hr, the peptide contents of the same samples were raised to about 85%.

In vitro measurement of LH and FSH releasing activities was performed as described previously,[27] and the results are summarized in Tables II and III.

Rat Anterior Pituitary Cyclic AMP Level. Cyclic AMP levels in rat anterior pituitary glands[28] were determined by

the competitive protein binding assay.[29] Decapeptide amide I exhibited marked increase of cyclic AMP concentration in rat anterior pituitary within 1 min after incubation. The minimum effective dose of I on cyclic AMP formation was 10 ng/ml. In the cases of II and III, no stimulation of cyclic AMP formation was observed.

Discussion

The above experimental evidence indicates that the synthetic LH-RH/FSH-RH (I) possesses a high degree of purity. The synthesis of I according to the route shown in Scheme I was repeated three times with identical results. Column chromatography on CM-Sephadex C-25 was shown to be very effective for the purification of I and II.

The hormonal activities of peptides I, II, and III were examined in comparison with pure natural LH-RH/FSH-RH. As can be seen in Table I, the potencies of the three preparations of our synthetic decapeptide amide I were the same as that of pure natural LH-RH/FSH-RH in the *in vivo* assay for LH-RH activity and the analogs II and III were inactive or, at best, very weakly active as described previously.[13] In the *in vitro* assay, II and III showed very low if any activity in a test based on the release and synthesis of LH and FSH in rat anterior pituitary cultures, while I possessed the same activity as that of natural LH-RH/FSH-RH. These demonstrate clearly that the N-terminal pGlu moiety plays an important role for biological function of LH-RH/FSH-RH.

In addition, observation on the effect of I, II, and III on rat anterior pituitary cyclic AMP levels indicated that decapeptide amide I stimulated cyclic AMP formation even at a concentration of 10 ng/ml, whereas II and III were inactive at dose of 50 μg/ml. This provides an additional support for the importance of pGlu moiety for the hormonal activity.

As mentioned by Hofmann, *et al.*,[30] numerous factors could be considered which may effect the apparent biological potency, when the activities of fragments of a peptide hormone are examined. However, the present investigation demonstrates conclusively that removal of pGlu moiety from the LH-RH/FSH-RH molecule results in nearly complete loss of the LH-RH and FSH-RH activities. Further investigation will be necessary to clarify whether pGlu moiety in the molecule is needed for the binding to the receptor or the functional effect.

Table I. *In Vivo* Assay for LH-RH Activity of Synthetic LH-RH/FSH-RH[a]

Sample	Dose, ng/rat	Plasma LH Level, ng/ml ± SE
Saline		6.1 ± 0.9
Natural LH-RH/FSH-RH	0.5	18.6 ± 3.0
	2.5	49.3 ± 11.5
Synthetic LH-RH/FSH-RH preparation 1	0.5	20.1 ± 4.5
	2.5	45.2 ± 5.6
Saline		6.4 ± 0.6
Natural LH-RH/FSH-RH	0.5	14.5 ± 1.4
	2.5	37.9 ± 2.5
Synthetic LH-RH/FSH-RH preparation 2	0.5	11.1 ± 1.7
	2.5	41.9 ± 11.5
Synthetic LH-RH/FSH-RH preparation 3	0.5	13.1 ±1.5
	2.5	55.3 ±1.6

[a]Potency: synthetic LH-RH/FSH-RH *vs.* pure natural LH-RH/FSH-RH (% LH-RH activity with 95% confidence limits assumed as 100%): preparation 1, 93% (28.6-278%); preparation 2, 98% (37.4-254%); preparation 3,150% (120-180%).

Experimental Section

Melting points were determined on a Mitamura Riken capillary melting point apparatus and are uncorrected. Microanalyses were performed by the Analytical Center of Shizuoka College of Pharmacy. All analytical samples gave combustion values for C, H, and N within 0.4% of the theoretical values. Peptides containing Arg(NO_2) moiety gave slightly low N value.[31] R_f^I and R_f^{II} values refer to the solvent systems: 1-BuOH-AcOH-H_2O (4:1:5) and 1-BuOH-pyridine-AcOH-H_2O (30:20:6:24), respectively. Optical rotations were measured on a Yanaco automatic polarimeter OR-50. Amino acid analyses were performed with a Hitachi Model KLA-3B amino acid analyzer. Acid hydrolysis of a sample for amino acid analysis was

Table II. Effect of Synthetic LH-RH/FSH-RH and Its Analogs on the Release and Synthesis of LH in Rat Anterior Pituitary Culture[a]

Sample	Dose of sample (total of 5 doses), μg/pit.	Total LH content (ng of LH/pit. ± SE) by radioimmunoassay[b] Medium	Tissue	Total	Increase
Control		832 ± 26	784 ± 15	1616	
Natural LH-RH/FSH-FH	0.02	2889 ± 105, p^c = 0.005	338 ± 24, p^c = 0.005	3227	1611
Synthetic LH-RH/FSH-RH (I) preparation 1	0.02	3213 ± 124, p^c = 0.001	296 ± 23, p^c = 0.005	3509	1893
des-pGlu[1]-LH-RH/FSH-RH (II)	2.00	1405 ± 142, p^c = 0.005	686 ± 28, p^c = 0.005	2091	475
des-pGlu[1]-des-His[2]-LH-RH/FSH-RH (III)	2.00	1378 ± 83, p^c = 0.001	1563 ± 41, p^c = 0.001	2941	1325

[a]5-Day tissue culture, TC-37-TWR. [b]Mean of six determinations. [c]Student's t test, stimulated *vs.* control.

Table III. Effect of Synthetic LH-RH/FSH-RH and Its Analogs on the Release and Synthesis of FSH in Rat Anterior Pituitary Culture[a]

Sample	Dose of sample (total of 5 doses), μg/pit.	Total FSH content (ng of FSH/pit. ± SE) by radioimmunoassay[b] Medium	Tissue	Total	Increase
Control		19,100 ± 546	3798 ± 133	22,893	
Natural LH-RH/FSH-RH	0.02	26,500 ± 957, p^c = 0.001	2030 ± 39, p^c = 0.001	28,530	5637
Synthetic LH-RH/FSH-RH (I) preparation 1	0.02	28,400 ± 400, p^c = 0.001	1423 ± 58, p^c = 0.001	29,823	6930
des -pGlu[1]-LH-RH/FSH-RH (II)	2.00	18,433 ± 789, p^c = NS	2814 ± 141, p^c = 0.001	21,247	−1000
des-pGlu[1]-des-His[2]-LH-RH/FSH-RH (III)	2.00	24,966 ± 754, p^c = 0.001	6883 ± 135, p^c = 0.001	31,849	8956

[a]5-Day tissue culture, TC-37-TWR. [b]Mean of six determinations. [c]Student's t test, stimulated *vs.* control.

conducted with 6 *N* HCl in the presence of phenol at 110° for 24 hr in an evacuated sealed tube. All solvents were of reagent grade and were distilled before use. Evaporations were carried out *in vacuo* at 40–45° in rotary evaporators. CM-Sephadex C-25 (Na form) was washed twice with 10% AcOH and then with H_2O before use.

Z-pGlu-OSu. DCI (4.54 g) was added to a solution of Z-pGlu-OH (5.25 g) and *N*-hydroxysuccinimde (2.30 g) in THF (50 ml) at 0°, and the mixture was stirred at 4° for 15 hr. After filtering, the solutions was evaporated to give an oil which was crystallized from petroleum ether (bp 30–60°). The material was recrystallized from *i*-PrOH: 5.60 g; yield 78%; mp 131–133°. *Anal.* ($C_{17}H_{16}N_2O_7$) C, H, N.

H-Pro-Gly-NH_2 · HBr (IV). Z-Pro-Gly-NH_2[7] (3.05 g) was dissolved in AcOH (7 ml) and 25% HBr in AcOH (10 ml) was added. The mixture was allowed to stand at room temperature for 1 hr. Anhydrous Et_2O was added, and the resulting H-Pro-Gly-NH_2 · HBr (IV) was collected by filtration, washed with Et_2O, and dried over KOH, R_f^I 0.16.

Z-Arg(NO_2)-Pro-Gly-NH_2 Hemihydrate (V). A solution of Z-Arg(NO_2)-OH[14] (3.53 g) and *N*-methylmorpholine (1.00 ml) in DMF (10 ml) was cooled to −15° and isobutyl chloroformate (1.30 ml) was added. After 1 min, the resulting mixed anhydride was combined with an ice-cooled solution of the above IV and Et_3N (2.70 ml) in DMF (8 ml). The mixture was kept at 0° for 5 min and at 15° for additional 30 min. The solvent was evaporated and the residue was dissolved in H_2O (50 ml). The solution was washed with three portions of EtOAc and then extracted with five portions of 1-BuOH which were washed with six portions of 2% AcOH. Evaporation of the solvent gave an oil which was solidified by addition of Et_2O. After precipitation from MeOH–EtOAc, pure V was obtained: 3.20 g; yield 62%; mp 92–94°; $[\alpha]^{28}D$ −39.7° (*c* 1.0, MeOH); R_f^I 0.54. *Anal.* ($C_{21}H_{30}N_8O_7 \cdot 0.5H_2O$) C, H, N.

Z-Leu-Arg(NO_2)-Pro-Gly-NH_2 (VI). Compound V (2.80 g) was decarbobenzoxylated in the manner as described for IV, R_f^I 0.15. Z-Leu-OSu[15] (2.40 g) was added to a solution of the above deblocked tripeptide amide HBr and Et_3N (1.40 ml) in DMF (25 ml). The mixture was left at room temperature for 15 hr. The solvent was evaporated and the residue was distributed between 1-BuOH and H_2O in the manner as described for V. Evaporation of 1-BuOH layers gave an oily residue which was solidified by addition of Et_2O. After precipitation from MeOH–EtOAc, pure VI was obtained: 3.30 g; yield 98%; mp 147–151° dec; $[\alpha]^{28}D$ −49.0° (*c* 1.0, MeOH); R_f^I 0.53 *Anal.* ($C_{27}H_{41}N_9O_8$),C, H, N.

Z-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (VII). Decarbobenzoxylation of compound VI (3.11 g) was performed in the manner as described for IV, R_f^I 0.32. Z-Gly-OSu[15] (1.85 g) was added to a solution of the above tetrapeptide amide HBr and Et_3N (1.40 ml) in DMF (30 ml). The mixture was left at room temperature for 15 hr. Pure VII was obtained in the manner as described for V: 2.71 g; yield 80%; mp 137–141° dec. An additional 0.55 g of VII was obtained from the mother liquor of the precipitation, mp 136–141° dec, which was combined to the first crop: $[\alpha]^{26}D$ −51.0° (*c* 1.0, MeOH); R_f^I 0.52. *Anal.* ($C_{29}H_{44}N_{10}O_9$) C, H; N: calcd, 20.70; found, 20.10.

Z-Ser-Tyr-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (VIII). Decarbobenzoxylation of compound VII (3.10 g) was performed in the manner as described for IV, R_f^I 0.24. Z-Ser-Tyr-N_2H_3[16] (2.86 g) was dissolved in DMF (30 ml) and cooled to −15°. To this solution were added 6 *N* HCl in dioxane (3.50 ml) and *i*-AmONO (1.10 ml). The mixture was left at −10° for 5 min and neutralized with Et_3N. An ice-cold solution of the above deblocked pentapeptide amide HBr and Et_3N (0.75 ml) in DMF (30 ml) was added. The mixture was stirred at −10° for 2 hr and at 4° for further 15 hr. Pure product VIII was isolated in the manner as described for V: 3.82 g; yield 90%; mp 178–181° dec; $[\alpha]^{28}D$ −52.3° (*c* 1.1, MeOH); R_f^I 0.48. *Anal.* ($C_{41}H_{58}N_{12}O_{13}$) C, H; N: calcd, 18.13; found, 17.32.

Z-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 Acetate (X). Compound VIII (3.80 g) was hydrogenated for 48 hr over Pd in MeOH (120 ml) and 10% AcOH (70 ml). The catalyst was removed by filtration, the filtrate was evaporated, and the residue was lyophilized to give IX, R_f^I 0.21. To a solution of the above hydrogenated heptapeptide amide IX and Et_3N (0.57 ml) in DMF (40 ml) was added Z-Trp-OTCP[19] (4.14 g). The mixture was left at room temperature for 36 hr. The desired pure X was obtained in the manner as described for V: 3.26 g; yield 70%; mp 182–185° dec; $[\alpha]^{28}D$ −39.4° (*c* 1.0, MeOH); R_f^I 0.48 *Anal.* ($C_{52}H_{69}N_{13}O_{12} \cdot CH_3COOH$) C, H, N.

H-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 Diacetate (des-pGlu[1]-des-His[2]-LH-RH/FSH-RH) (III). Compound X (3.10 g) was hydrogenated for 48 hr over Pd in MeOH (100 ml) and 10% AcOH (25 ml). After filtering and evaporating the solvents, the residue was lyophilized from a small volume of H_2O, and precipitation from MeOH–EtOAc gave pure III: 2.84 g; yield 98%; $[\alpha]^{28}D$ −36.1° (*c* 1.0, 1 *M* AcOH); amino acid ratios in acid hydrolysate, $Ser_{0.91}Tyr_{0.98}Gly_{2.01}Leu_{1.00}Arg_{0.97}Pro_{1.02}$ (88% average recovery). This material was used for the coupling in the next process.

Z-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 Diacetate Dihydrate (XI). A solution containing the azide, prepared from Z-His-N_2H_3[20] (1.73 g), 6 *N* HCl in dioxane (2.85 ml), and *i*-AmONO (0.77 ml) in the manner as described for VIII, was added to an ice-cold solution of the above III and Et_3N (0.4 ml) in DMF (25 ml). The mixture was stirred at −10° for 3 hr and at 4° for additional 24 hr. Evaporation of the solvents gave an oily material which was solidified by addition of EtOAc. The product was collected, dried, and lyophilized from a small amount of H_2O, which was then dissolved in H_2O (600 ml). The solution was applied to a column of CM-Sephadex C-25 (3 × 10 cm), which was eluted successively with H_2O (800 ml) and NH_4OAc buffer: 0.05 *M* (800 ml), 0.08 *M* (600 ml), 0.1 *M* (1000 ml), and finally 1 *M* AcOH (5000 ml). The 0.1 *M* NH_4OAc and 1 *M* AcOH eluates containing the desired material were pooled and the solvent was evaporated. The residue was lyophilized three times, and the product dissolved in 1 *M* AcOH was desalted by a Bio-Gel P-2 column (4 × 45 cm) with 1 *M* AcOH as eluent: 3.36 g; yield 90%; $[\alpha]^{28}D$ −44.3 °(*c* 1.1, MeOH); R_f^I 0.41, R_f^{II} 0.71. *Anal.* ($C_{58}H_{76}N_{16}O_{13} \cdot 2CH_3COOH \cdot 2H_2O$) C, H, N.

H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 Triacetate (des-pGlu[1]-LH-RH/FSH-RH) (II). Compound XI (1.86 g) was hydrogenated in the manner as described for III. The product was obtained quantitatively: $[\alpha]^{28}D$ −30.6° (*c* 1.0, 1 *M* AcOH); R_f^I 0.20, R_f^{II} 0.68; amino acid ratios in acid hydrolysate, $His_{0.98}Ser_{0.92}Tyr_{1.01}Gly_{2.02}Leu_{0.99}Arg_{0.97}Pro_{1.03}$ (83% average recovery); amino acid ratios in AP-M digest, $His_{1.03}Ser_{0.94}Tyr_{0.98}Gly_{2.02}Leu_{1.03}$ (77% average recovery) (Trp was not estimated and Arg and Pro were not found).

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 Diacetate Pentahydrate (LH-RH/FSH-RH) (I). Z-pGlu-OSu (0.74 g) was added to a solution of II, prepared from XI (1.86 g), and Et_3N (0.04 ml) in DMF (50 ml). The mixture was left at room temperature for 20 hr. Additional Z-pGlu-OSu (0.20 g) was added. After standing for 2 hr, the solvent was removed and the residue was dissolved in H_2O (50 ml), which was washed with three portions of EtOAc. The aqueous solution was then submitted to hydrogenation for 30 hr over Pd in the presence of 10% AcOH (30 ml). After filtering and evaporating the solvent, the residue was lyophilized from a small volume of H_2O. The resulting material was dissolved in H_2O (800 ml) and the solution was applied to a CM-Sephadex C-25 column (3 × 10 cm), which was eluted successively with H_2O (800 ml) and NH_4OAc buffer: 0.05 *M* (400 ml), 0.08 *M* (800 ml), 0.1 *M* (600 ml), 0.125 *M* (1800 ml), and 0.3 *M* (1000 ml). The 0.1 and 0.125 *M* eluates containing the desired material were pooled, the solvent was removed by evaporation, and the residue was lyophilized three times from a small volume of H_2O. The product was completely desalted by a Bio-Gel P-2 column using 1 *M* AcOH as eluent: 1.42 g; yield 75%; $[\alpha]^{28}D$ −50.5° (*c* 1.0, 1 *M* AcOH); R_f^I 0.23, R_f^{II} 0.70; amino acid ratios in acid hydrolysate $Glu_{1.01}His_{0.93}Ser_{0.91}Tyr_{1.03}Gly_{2.03}Leu_{1.01}Arg_{1.06}Pro_{0.94}$ (78% average recovery); Tyr/Trp = 0.96.[24] *Anal.* ($C_{55}H_{75}N_{17}O_{13} \cdot 2CH_3COOH \cdot 5H_2O$) C, H, N.

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Catechol *O*-Methyltransferase. 1. Kinetics of Tropolone Inhibition

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In an attempt to clarify the mechanism by which tropolones inhibit catechol *O*-methyltransferase (COMT), kinetic studies have been performed on COMT purified from rat liver. Rate studies indicate that tropolone is a linear noncompetitive inhibitor with respect to 3,4-dihydroxybenzoic acid (K_{ii} = 247 μM, K_{is} = 22 μM) and an uncompetitive inhibitor with respect to *S*-adenosyl-L-methionine; a complex relationship exists with respect to magnesium. Similar results were observed with *l*-norepinephrine and 3,4-dihydroxyacetophenone as substrates, as well as with 4-methyltropolone and β-thujaplicin as inhibitors. The patterns of inhibition and the kinetic parameters for tropolone inhibition remained constant through various stages of enzyme purification. The effect of pH on the substrate kinetic parameters (K_m and V_{max}) and tropolone inhibition parameters (K_{is} and K_{ii}) was also investigated. The ability of tropolone to inhibit COMT was found to decrease with increasing pH which can be attributed to an increase in K_{is}. This probably reflects the ionization of tropolone rather than dissociation of a group on the enzyme. The K_{ii} for tropolone was observed to decrease with increasing pH. The significance of this data relative to the mechanism of O-methylation by COMT is discussed.

The inactivation of catecholamines and the detoxification of many zenobiotic catechols is dependent upon the enzyme catechol *O*-methyltransferase (COMT) (E.C. 2.1.1.6).† COMT is a soluble, magnesium-requiring enzyme which transfers a methyl group from *S*-adenosylmethionine (SAM) to a catechol substrate resulting in the formation of the meta and para O-methylated derivatives.[1,2] Because of the importance of COMT in the extraneuronal inactivation of norepinephrine, inhibition of this route of metabolism has generated considerable research interest.[3-10]

Several classes of synthetic inhibitors of COMT have been identified: (a) substrates such as catechols,[3] desmethylpapaverine,[4] *o*-dihydroxyphenylacetamides,[5] and pyrogallol;[6] (b) product type inhibitors such as 3-hydroxy-4-methoxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3,5-dihydroxy-4-methoxybenzoic acid,[7] and *S*-adenosylhomocysteine;[8] and (c) dead-end inhibitors such as tropolones[9] and pyridoxal 5′-PO_4.[10]

Since dead-end inhibitors have proven useful in the elucidation of certain enzyme mechanisms,[11] and since the kinetic mechanism of COMT appears to be the subject of considerable controversy,[12-14,‡] it was felt that an extensive investigation of the kinetics of tropolone inhibition on COMT could provide information relative to the mechanism of methyl transfer. The present paper reports the results of such an investigation and provides information about the kinetic mechanism for COMT.

Results and Discussion

In Vitro Inhibition Patterns. The inhibition patterns toward COMT for three tropolone derivatives were determined using plots of reciprocal velocities against reciprocals of the substrate concentrations. As shown in Figure 1, a noncompetitive type of inhibition was observed when DHB was the variable substrate and tropolone was the inhibitor. Furthermore, this inhibition pattern can be classified as linear noncompetitve since replots of the slopes and intercepts *vs.* inhibitor concentrations are linear (Figure 2).[15] This type of linear relationship with a dead-end inhibitor provides evidence against the possibility that two or more molecules of the inhibitor add simultaneously to the same form of the enzyme.[15] This, however, does not rule out the possibility that tropolone may be combining with two different forms of the enzyme producing the noncompetitive

†Abbreviations used are: SAM, *S*-adenosyl-L-methionine; DHB, 3,4-dihydroxybenzoic acid; *l*-NE, *l*-norepinephrine; DHA, 3,4-dihydroxyacetophenone; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.

‡C. Creveling, personal communication.