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An Improved Solid-Phase Synthesis of the Luteinizing-Hormone Releasing-Hormone/Follicle-Stimulating Hormone Releasing-Hormone (LH-RH/FSH-RH)†

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The decapeptide <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I) has been reported by Schally[1] as the structure of the luteinizing-hormone releasing-hormone/follicle-stimulating hormone releasing-hormone (LH-RH/FSH-RH). Of the syntheses of I reported thus far[2-7] some give no details and others result in low to moderate yields of hormone and the methodology involved is mostly not convenient for the synthesis and purification of substantial quantities of pure I.

In a synthesis employed previously in this laboratory[8] the triprotected decapeptide <Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (II) was prepared in 10% yield by solution methods employing mainly the active ester method[9] and in 35-40% yield by the solid-phase method.[10] From II, the decapeptide I was obtained in about 30% yield.

In this paper we describe attempts to improve the synthesis of I employing the tosyl group to protect the side chain functionalities of histidine[11] and of arginine[12] during the solid-phase synthesis. In one procedure, starting with Boc-glycine-resin, the peptide was assembled by the procedure of Stewart and Woolley,[13] employing 4 *M* HCl-dioxane and *tert*-butyloxycarbonylamino acids (4 mol excess), with *tert*-butyloxycarbonylhistidine (Tos) the derivative chosen to introduce histidine, and 1% mercaptoethanol employed to prevent destruction of tryptophan during acid treatment of tryptophan-containing peptides.[14] The completed peptide-resin was ammonolyzed to yield the triprotected decapeptide amide II in 30% yield. The use of the N^{Im}-tosyl derivative does not appear to lead to an improvement in yield of II when compared to our previous synthesis with *tert*-butyloxycarbonylhistidine.

In a more successful solid-phase synthesis of I, the protected decapeptide <Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH_2 (III) was prepared in 52% yield. The methods employed were the same as those described for the solid-phase synthesis of II, with the exception that *tert*-butyloxycarbonylarginine (Tos) was the derivative employed to introduce the arginyl residue. The material obtained after ammonolysis of the triprotected peptide resin was of higher purity than the product previously obtained by the method employing *tert*-butyloxycarbonylarginine (NO_2), and it was easier to purify by column chromatography on silica gel. The peptide-resin was also cleaved by methanolysis with MeOH-TEA and the intermediate triprotected decapeptide methyl ester obtained was then ammonolyzed to III. This method gave no significant change in yield but did produce a cleaner, more easily purified product. The purified tosyl-decapeptide III was deprotected with HF-anisole[15] and the crude product purified by gel filtration[16] on Sephadex G-25. The main product obtained was subjected to a second gel filtration, yielding the pure free peptide I in 50% conversion from III. Thus, the synthesis of I *via* the tosyl-decaptide III leads to an overall yield of about 26% of I from glycine-resin, as compared to that *via* the nitro-decapeptide II which leads to an overall of I of about 10%.

The synthetic LH-RH was assayed *in vivo* for LH release in estrogen-progesterone-treated rats and *in vitro* for LH and FSH release after incubation of normal rat pituitaries. The methodology employed in these various assays has been previously described.[17] The results of these various assays (Table I) show that synthetic LH-RH is at least as active in releasing LH and FSH as the highly purified natural LH-RH sample AVS-77-33 No. 215-269‡ within the confidence limits shown.

Thus, the synthetic methodology described here is promising for obtaining I with excellent yields and quality. The preparation of gram quantities of this hormone for extensive biological studies becomes greatly simplified.

Experimental Section§

<Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (II). A sample of 3.57 g of Boc-glycine-polymer[8] (0.4 mmol of gly/g) was taken manually through nine coupling cycles by the solid-phase method[13] as described in our previous synthesis of LH-RH/FSH-RH.[8] In each cycle the Boc group was removed with 4 *M* HCl-dioxane, and after neutralization with TEA coupling was accomplished with a 4 mol excess of the desired Boc-amino acid and DCC. On the eighth coupling cycle Boc-His(Tos) was employed to introduce the histidyl residue. The completed decapeptide resin, 5.39 g, was cleaved in 100 ml of DMF-MeOH (1:1) saturated with NH_3 for 24 hr at room temperature. After filtration and evaporation of the solvents *in vacuo*, the crude product ob-

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§All optically active amino acids are of the L configuration. A Thomas-Hoover apparatus was used for melting point determinations in capillary melting tubes. Where analyses are indicated only by symbols of the elements, analytical results were obtained for the elements within ±0.4% of the theoretical value. Where tlc was used to determine purity of intermediates and products, silica gel G plates were used; the solvent system employed was 33% MeOH-$CHCl_3$ with Pauly, Ehrlich, and Cl_2-tolidine color reactions. Thin layer electrophoresis was performed on Quanta-gram Industries Q2 cellulose (250M) with a Desaga-Brinkman apparatus at 400 V for 2 hr. For amino acid analysis, the peptide was hydrolyzed in 6 *M* HCl containing 0.1 *M* phenol (1% v/v) for 18 hr in an evacuated tube at 100°; the analysis was done with a Beckman Model 120-B amino acid analyzer. The nmr spectra were obtained at 100 MHz using a Varian Associates HA-100 spectrometer. The following abbreviations were used throughout the text: DCC = dicyclohexylcarbodiimide, Py = pyridine, Boc = *tert*-butyloxycarbonyl, TEA = triethylamine.

Table I. Effect of Synthetic Decapeptide on *in Vivo* and *in Vitro* FSH and LH Release in Rats

Assay no.	Method	Pituitary hormone assay used	Rel potency[a]	95% limits	Precision index
1	*In vivo*	LH (RIA)[c]	111	59–210	
2	*In vitro*[b]	FSH[d]	193	65–763	0.32
		FSH (RIA)[e]	114	91–143	
		LH (RIA)[c]	137	110–171	
3	*In vitro*[b]	LH[f]	120	43–195	0.11
		FSH (RIA)[e]	197	160–248	
		LH (RIA)[c]	142	62–275	
Mean potency			145		

[a]As per cent pure natural fraction, AVS-77-33 No. 215-269. [b]Rat hemipituitaries.[17] [c]G. D. Niswender, A. R. Midgley, Jr., S. E. Monroe, and L. E. Reichert, Jr., *Proc. Soc. Exp. Biol. Med.*, 128, 807 (1968). [d]S. L. Steelman and F. M. Pohley, *Endocrinology*, 53, 604 (1953). [e]M. L. Hermite, G. D. Niswender, L. E. Reichert, Jr., and A. V. Nalbandov, *ibid.*, 84, 1166 (1969). [f]A. F. Parlow in "Human Pituitary Gonadotropin," A. Albert, Ed., Charles C Thomas, Springfield, Ill., 1961, p 300.

tained was purified by column chromatography on silica gel (100 g) employing 5–15% MeOH–$CHCl_3$ to remove faster moving impurities and 33% MeOH–$CHCl_3$ to elute the desired product. The eluent was monitored by tlc. Appropriate fractions were combined and evaporated to a solid residue which was dissolved in hot MeOH. The solid which separates was collected yielding 0.6 g: mp 169° dec; $[\alpha]^{25}D$ −26.9° (*c* 1, AcOH) [lit.[8] mp 166–169°; $[\alpha]^{25}D$ −25.2° (*c* 1, AcOH)]. The nmr spectrum (CD_3COOD) was consistent with the structure of II and identical with the nmr of a sample of II made previously.[8] *Anal.* ($C_{69}H_{86}N_{18}O_{15}$) C, H, N.

<Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-NH_2 (III). A sample of 3.57 g of Boc-Gly-polymer (0.4 mmol of Gly/g of polymer) was taken through nine cycles of deprotection and coupling to give the desired protected decapeptide polymer by the methods described in the preceding experiment, except for the use of Boc-Arg(Tos) in the coupling step of the second cycle. The protected decapeptide polymer was suspended in MeOH (240 ml) and TEA (13.9 ml) for 20 hr at room temperature. The suspension was filtered and the resin resuspended in identical amounts of MeOH and TEA for an additional 30 hr. After filtration the clear solution was evaporated *in vacuo*. The yield of crude product was 3.34 g of material which showed one main component as detected by tlc.

The nmr spectrum was consistent with the structure showing a sharp characteristic singlet (δ 3.7 assigned to methyl ester O–CH_3 protons). This material was dissolved in MeOH (30 ml) and carefully added to liquid NH_3 (30 ml) and the resulting solution was allowed to stand overnight. After removal of the solvents *in vacuo*, the crude product was purified by column chromatography on 200 g of silica gel with 5–15% MeOH–$CHCl_3$ to elute fast-moving impurities and 33% MeOH–$CHCl_3$ to elute the desired product. Appropriate fractions selected from the tlc profile of all fractions (R_f 0.3) were combined and evaporated to a solid. The purified product was dissolved in hot MeOH and the clear solution allowed to cool slowly to room temperature. The white solid obtained was collected yielding 1.12 g: mp 164–169°; $[\alpha]^{25}D$ 26.0° (*c* 1, AcOH). The nmr spectrum showed the loss of the methyl ester O–CH_3 protons and was consistent with the structure. *Anal.* ($C_{76}H_{93}N_{17}O_{15}S$) H, N; C: calcd, 60.2; found, 59.7.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I). A 0.500-g sample of the protected decapeptide was cleaved with 10 ml of liquid HF and 1 g of anisole for 1 hr at 0°. Excess acid was flushed out with N_2 and the residue washed with Et_2O. This crude product was dissolved in 10% AcOH and the solution was washed in a separatory funnel with Et_2O and lyophilized. The white powder obtained was dissolved in 0.1 *M* AcOH and the solution passed slowly through ion exchange resin AG1-X2 (AcO^-) to remove F^- and lyophilized to yield 352 mg (90%). A sample of this crude product (201 mg) was purified by gel filtration in 0.1 *M* AcOH on a Sephadex G-25 column (110 × 2.4 cm). Fractions corresponding to the central portions of the main peak (280 nm) were combined and lyophilized. This material was chromatographed once again through Sephadex G-25 yielding one main symmetrical peak (280 nm). Fractions corresponding to the central portions of the peak were combined and lyophilized to yield 113 mg (50%) of white fluffy powder. A two-dimensional thin-layer electrophoresis with 0.1 *M* pyridine–AcOH, pH 6.5, followed by ascending tlc with *n*-BuOH–AcOH–H_2O (4:1:1) gave after spraying with Pauli, Ehrlich, and Sakaguchi spray a single spot in a position similar to that of a standard treated under the same condition.

The amino acid analysis[18] following acid hydrolysis showed the following amino acid ratios:[#] Gly 2.04, Pro 1.00, Arg 1.00, Leu 1.01, Tyr 0.98, Ser 0.93, His 1.00, Glu 1.08, and Trp 0.94. The optical rotation $[\alpha]^{25}D$ −50.5° (*c* 1, 1%AcOH) was similar to that found in a previous synthesis,[8] $[\alpha]^{24}D$ −50.5° (*c* 1, 1% AcOH). *Anal.* ($C_{55}H_{75}N_{17}O_{13}\cdot 2CH_3COOH\cdot 4H_2O$) C, H, N, O.

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[#]Thioglycolic acid (1%) was added to prevent tryptophan breakdown during acid hydrolysis.