sulsai Zasshi, 14, 457 (1966).
(8) T. Ueda, Y. Okamoto, T. Tsuji, and M. Muraska, *Chem. Pharm. Bull.*, **16**, 2355 (1968).
(9) J. P. Greenstein, S. M. Birnbaum, and M. C. Otey, *J. Amer. Chem. Soc.*, **75**, 1994 (1953).
(10) T. Kato, A. Tuksada, and T. Ueda, *Chem. Pharm. Bull.*, **20** (5), 901 (1972).
(11) A. W. Dox, "Organic Syntheses," Collect. Vol. I, Wiley, New York, N. Y., 1932, p 5.

Synthesis of Bradykinin *via* Oximinopyrazoline Active Esters (OPmp)†

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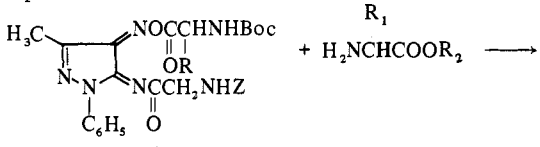
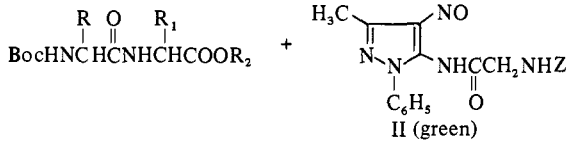
The present communication reports a stepwise synthesis of bradykinin, employing oximinopyrazolineamino acid active esters (OPmp, I).[2a] These esters were allowed to react with amino acid or peptide esters according to the following equation.[2-4]



I (red)



II (green)

The synthesis was carried out *via* a stepwise procedure; protection of functional groups was planned in view of a final cleavage with HF.[5] The masking of the serine hydroxyl group could be avoided since no reaction of OPmp active esters upon such a function has been observed.[3] Very high rates (between 2 and 4 min) and satisfactory yields were achieved in the formation of all peptide bonds.

At each stage, the peptide was isolated from the nitrosoacylamine II, taking advantage of the fact that the former is insoluble in ethyl ether whereas the latter is soluble in the same solvent. After deprotection with HF at 0° for 30 min, the bradykinin trifluoride was desalted by passing its aqueous solution through a column of Amberlite CG 400; the product was then converted to the acetate form and lyophilized. The nonapeptide had optical rotation and elemental and amino acid analyses corresponding to those of the authentic product;[5,6] its biological activity was tested in comparison with a sample of bradykinin taken as standard.[7]

Experimental Section

Melting points were taken on a Tottoli capillary melting point apparatus and are uncorrected. Where analyses are indicated by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. Times required for complete condensation to occur were 4 min for protected peptides 1, 4, and 8 and 2 min for protected peptides **2**, **3**, **5**, **6**, and **7**. The intermediates reported below were recrystallized from the appropriate solvents until homogeneous at tlc in two different solvent systems: 1-butanol–glacial acetic acid–water (3:1:1); ethyl acetate–pyridine–glacial acetic acid–water (60:20:6:14).

Boc-Phe-Arg(NO_2)-OBzl (1). To a solution of Arg(NO_2)-OBzl[8] (1.54 g, 5 mmol) in chloroform (60 ml), the red active ester Boc-Phe-OPmp[4] (3.20 g, 5 mmol) was added. When the solution became green, it was evaporated; the residue was triturated with ethyl ether and then crystallized from ethyl acetate–ethyl ether to yield 2.20 g (80%) of 1: mp 156–157°; $[\alpha]^{25}_{578}$ −18.93° (*c* 0.48, $CHCl_3$) *Anal.* ($C_{27}H_{36}N_6O_7$) C, H, N.

Boc-Pro-Phe-Arg(NO_2)-OBzl (2). Compound 1 (1.66 g, 3 mmol) was allowed to react with TFA (5 ml) for 20 min at room temperature; the resulting tripeptide trifluoroacetate was precipitated and washed with ethyl ether and then dried *in vacuo* over sodium hydroxide. A solution of the salt (1.71 g, 3 mmol) in chloroform (60 ml) was treated with triethylamine (0.42 ml, 3 mmol) and Boc-Pro-OPmp[4] (1.70 g, 3 mmol). After 2 min the green solution was washed with citric acid (5%) and water and dried over Na_2SO_4 Evaporation gave a solid that was triturated with ether and crystallized from ethyl acetate–ethyl ether to give **2** (1.57 g, 80%): mp 86–89°; $[\alpha]^{25}_{578}$ −48.06° (*c* 0.69, $CHCl_3$). *Anal.* ($C_{32}H_{43}N_7O_8$) C, H, N.

Using the above procedure for the subsequent steps of cleavage and condensation, the stepwise peptide elongation was carried out by means of molar amounts of OPmp active esters[4] of the required amino acid; the following protected peptides were obtained.

Boc-Ser-Pro-Phe-Arg(NO_2)-OBzl (3): yield 81%; mp 99–100°; $[\alpha]^{25}_{578}$ −39.15° (*c* 0.75, ethyl acetate). *Anal.* ($C_{35}H_{48}N_8O_{10}$) C, H, N. This and the following intermediates were recrystallized from ethyl acetate–ethyl ether.

Boc-Phe-Ser-Pro-Phe-Arg(NO_2)-OBzl (4): yield 82%; mp 113–116°; $[\alpha]^{25}_{578}$ −42.16° (*c* 0.53, ethyl acetate). *Anal.* ($C_{44}H_{59}O_{11}N_9$) C, H, N.

Boc-Gly-Phe-Ser-Pro-Phe-Arg(NO_2)-OBzl (5): yield 85%; mp 117–121°; $[\alpha]^{25}_{578}$ −40.39° (*c* 0.59, DMF). *Anal.* ($C_{46}H_{60}N_{10}O_{12}$) C, H, N.

Boc-Pro-Gly-Phe-Ser-Pro-Phe-Arg(NO_2)-OBzl (6): 86%; mp 119–122°; $[\alpha]^{25}_{578}$ −49.12° (*c* 0.58, DMF). *Anal.* ($C_{51}H_{67}N_{11}O_{13}$) C, H, N.

Boc-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg(NO_2)-OBzl (7): yield 84%; mp 127–132°; $[\alpha]^{25}_{578}$ −45.89° (*c* 0.50, DMF). *Anal.* ($C_{36}H_{74}O_{12}N_{14}$) C, H, N.

Boc-Arg(NO_2)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg(NO_2)-OBzl (8): yield 79%; mp 138–142°; from chloroform–ethyl ether; $[\alpha]^{25}_{578}$ −43.57° (*c* 0.50, DMF). *Anal.* ($C_{62}H_{85}N_{17}O_{17}$) C, H, N.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH · 3CH_3COOH (Bradykinin Triacetate). The protected nonapeptide 8 (0.134 g, 0.1 mmol) was placed in a HF reaction cylinder with anisole[5,9] (0.11 ml, 10 equiv) and HF (5 ml) was added. The mixture was allowed to react at 0° for 30 min with stirring. Excess HF was removed under reduced pressure at 0° and then with introducing a nitrogen stream. The residue of the reaction was dissolved in water (10 ml); the solution was extracted with ethyl ether to eliminate anisole, passed through a column (0.8 × 9 cm) of Amberlite CG 400 (OH^-) type II, and eluted with distilled water (about 40 ml) until the pH of the washing became 7. The eluate was collected in a flask containing acetic acid (2 ml) and lyophilized; 93 mg (70%) of product as triacetate pentahydrate was obtained: mp 162–170°; $[\alpha]^{20}D$ −81.1° (*c* 0.293, water). *Anal.* ($C_{50}H_{73}N_{15}O_{11}\cdot 3CH_3COOH\cdot 5H_2O$) C, H, N.

Its homogeneity was tested by paper electrophoresis (pH 3.5 and 8.5) and tlc (BuOH–AcOH–H_2O, 2:1:1) using ninhydrin and chlorine reagents. Amino acid analysis after hydrolysis (6 *N* HCl) gave Arg 1.96, Pro 2.96, Gly 1.00, Phe 1.94, and Ser 1.00. Biological activities in comparison with a sample of bradykinin (Sandoz), taken equal to 100, were as follows: rat uterus stimulation 100–120; guinea-pig ileum stimulation 90–100; dog hypotensive effect 100–110; rat duodenum inhibition 100–105.[7]

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References

(1) IUPAC-IUB Commission on Biological Nomenclature, *Eur. J. Biochem.*, 1, 375 (1967).
(2) (a) M. Guarneri, P. Giori, and C. A. Benassi, *Tetrahedron Lett.*,

†OPmp = 1-phenyl-3-methyl-4-oximino-5-(*N*-Z-glycyl)imino-2-pyrazoline esters. The following abbreviations are also used (see ref 1): Z = benzyloxycarbonyl; Boc = *tert*-butyloxycarbonyl; OBzl = benzyl ester; TFA = trifluoroacetic acid; DMF = dimethylformamide.

8, 665 (1971); (b) M. Guarneri, P. Giori, R. Ferroni, R. Tomatis, and C. A. Benassi, *Gazz. Chim. Ital.*, in press.
(3) M. Guarneri, A. Guggi, R. Tomatis, P. Giori, and C. A. Benassi, *ibid.*, in press.
(4) R. Tomatis, E. Menegatti, A. Guggi, M. Guarneri, R. Rocchi, and C. A. Benassi, *ibid.*, in press.
(5) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jap.*, **40**, 2164 (1967).
(6) E. D. Nicolaides and H. A. De Wald, *Nature (London)*, **187**, 773 (1960).
(7) G. Bertaccini and P. Zamboni, *Arch. Int. Pharmacodyn.*, **133**, 138 (1961).
(8) S. Sakakibara and N. Inukai, *Bull. Chem. Soc. Jap.* **39**, 1567 (1966).
(9) J. Lenard, *J. Org. Chem.* **32**, 250 (1967).

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.