Retrobradykinin: a Peptide with the Reverse Sequence of Bradykinin

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Retrobradykinin, the nonapeptide L-arginyl-L-phenylalanyl-L-prolyl-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolyl-L-arginine, which has the reverse sequence of bradykinin, was synthesized. The azide of N-carbobenzoxy-L-seryl-L-phenylalanylglycine reacted with methyl L-prolyl-L-prolylnitro-L-argininate to form methyl N-carbobenzoxy-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolylnitro-L-argininate. After removal of the carbobenzoxy protecting group, the latter was treated with nitrophenyl carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-prolinate to form methyl carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-prolyl-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolylnitro-L-argininate. Removal of the blocking groups by saponification and hydrogenation produced the free nonapeptide which showed less than 1/80,000 the activity of bradykinin on the isolated rat uterus.

In studies of synthetic analogs of biologically active peptides, functional groups have been altered, usually by acylation or alkylation and amino acid residues have been replaced or eliminated. However, the effect of substantial rearrangement of a peptide sequence on biological activity has not been studied to this time. Of particular interest appeared to be the reversal of a peptide sequence and bradykinin,[1a,b] a biologically highly active nonapeptide was chosen because of the considerable degree of symmetry inherent in this molecule. The resulting substance was termed retrobradykinin.[2a,b]

A comparison of the structure of bradykinin and its retro-analog placed in antiparallel juxtaposition (Figure 1) shows that the relative position of R groups is, of course, the same in the two-dimensional sense. A comparison of the structures in parallel juxtaposition (Figure 2) shows that the amino acid residues at positions 1, 3, 5, 7, and 9 are identical in both bradykinin and retrobradykinin, demonstrating the high degree of symmetry in the bradykinin molecule.

It was observed however, that in the *in vitro* quiescent rat uterus assay,[3] retrobradykinin had no kinin activity at levels up to 80 γ/ml., while a fraction of a nonogram per ml. of bradykinin will provoke an observable and reproducible uterine response under the same conditions. Vogler, Lanz, and Lergier have also reported that this compound is inactive.[2b] Pre-incubation of rat uterine muscle with retrobradykinin in no way inhibited the response of the muscle to bradykinin, even in the presence of a three-thousand-fold excess of the retro-analog.

In the synthesis of retrobradykinin a sequence of steps was chosen specifically to preclude racemization (Figure 3). The three suitably protected tripeptide intermediates II, IV, VIII were prepared by stepwise acylation, utilizing the well established protection against racemization conferred by the N-carbobenzoxy protecting group.[4] For the same reason, the C-terminal moieties of the activated acyl tripeptides were glycine (Va) and proline (IX).

The protected tripeptide methyl carbobenzoxy-L-prolyl-L-prolylnitro-L-argininate (I) was prepared by the reaction of a mixed anhydride of carbobenzoxy-L-prolyl-L-proline with methyl nitro-L-argininate. Exposure of I to a saturated solution of hydrobromic acid in acetic acid followed by trituration with ether, formed the hydrobromide of methyl L-prolyl-L-prolylnitro-L-argininate (II), which was utilized without purification.

The tripeptide derivative ethyl N-carbobenzoxy-O-acetyl-L-seryl-L-phenylalanylglycinate (IV) was prepared in a stepwise manner starting with ethyl glycinate and the nitrophenyl ester of carbobenzoxy-L-phenylalanine. Ethyl L-phenylalanylglycinate hydrobromide (III) was obtained from the acylated dipeptide ester by treatment with a saturated solution of hydrobromic acid in acetic acid. This dipeptide ester was in turn acylated with nitrophenyl N-carbobenzoxy-O-acetyl-L-serinate.[5] Exposure of the tripeptide derivative formed (IV) to methanolic hydrazine for twenty-four hours produced the hydrazide of N-carbobenzoxy-L-seryl-L-phenylalanylglycine (V). As expected, the O-acetyl moiety was removed by this treatment. Solution of V in a mixture of acetic acid and 1 *N* hydrochloric acid followed by the addition of sodium nitrite produced the water-insoluble azide (Va), which reacted with II in dimethylformamide in the presence of triethyl amine to form the acylated hexapeptide ester methyl N-carbobenzoxy-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolylnitro-L-argininate (VI). This amorphous hexapeptide was isolated in chromatographically and analytically pure form.

After exposure of compound VI to a saturated

(1) (a) M. Rocha e Silva, W. D. Beraldo, and G. Rosenfeld, *Am. J. Physiol.*, **156**, 261 (1949); (b) For review *cf.* E. Werle, *Angew Chem.*, **73**, 689 (1961).

(2) (a) The synthesis of retrobradykinin and 6-glycine bradykinin was announced at the New York Academy of Science Conference on Structure and Function of Biologically Active Peptides: Bradykinin, Kallidin and Congeners, March 22, 1962, by M. Bodanszky, M. A. Ondetti, J. T. Sheehan, and S. Lande, and also by (b) K. Vogler, whose work was subsequently published: K. Vogler, P. Lanz, and W. Lergier, *Helv. Chim. Acta*, **45**, 561 (1962).

(3) Pharmacology Department Manual, Emory University, Atlanta, Ga., 1951, p. 39.

(4) M. B. North and G. T. Young, *Chem. Ind.* (London), 1597 (1955); J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **74**, 6137 (1952).

(5) M. A. Ondetti, *J. Med. Pharm. Chem.*, in press.

Bradykinin H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
Retrobradykinin HO-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-H

Figure 1

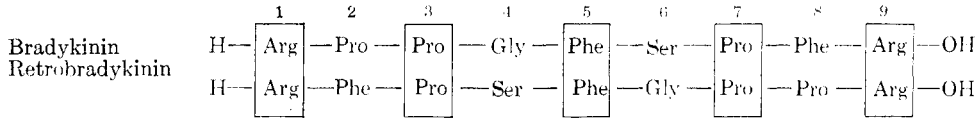

Figure 2

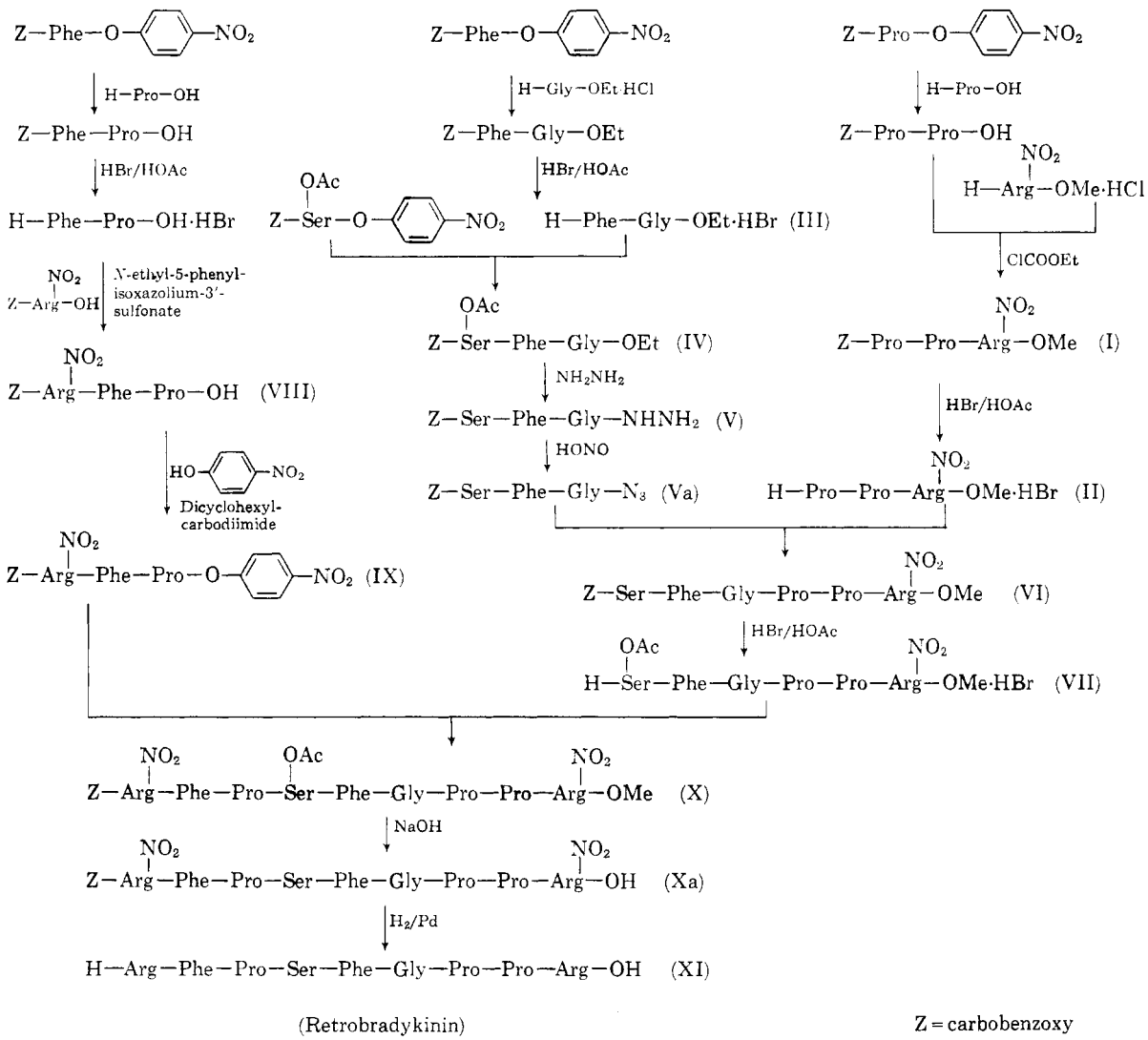

Figure 3

solution of hydrobromic acid in acetic acid for one or two hours, paper chromatography indicated the presence of two ninhydrin positive components. This inhomogeneous hydrobromide, methyl O-acetyl-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolyl-nitro-L-argininate (VII)[6] was used without purification in the subsequent step.

Carbobenzoxy-L-phenylalanyl-L-proline was prepared by acylation of L-proline with nitrophenyl carbobenzoxy-L-phenylalaninate. The protected dipeptide was then treated with a solution of hydrobromic acid in acetic acid to produce L-phenylalanyl-L-proline hydrobromide. This dipeptide reacted with the enol ester formed from carbobenzoxynitro-L-arginine and N-ethyl-5-phenylisoxazolium-3'-sulfonate[7] in dimethylformamide to give

(6) O-Acetylation of serine in anhydrous acetic acid containing hydrogen chloride was described by J. C. Sheehan, M. Goodman, and G. P. Hess, *J. Am. Chem. Soc.*, **78**, 1367 (1956). In our case, apparently the reaction did not proceed to completion.

(7) R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Am. Chem. Soc.*, **83**, 1010 (1961).

carbobenzoxynitro - L - arginyl - L - phenylalanyl-L-proline (VIII) which was isolated in crystalline form. Esterification of VIII with nitrophenol produced the crystalline nitrophenyl carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-prolinate (IX) in good yield.

The acyl tripeptide nitrophenyl ester, IX, was allowed to react with a slight excess of the hexapeptide ester hydrobromide, VII, in the presence of triethylamine. The resultant protected nonapeptide derivative (X) was exposed to aqueous sodium hydroxide to yield the acyl nonapeptide carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-prolyl-L-seryl-L - phenylalanylglycyl - L - prolyl - L - prolylnitro-L-arginine (Xa). A portion of this material in its crude form was hydrogenated over a palladium catalyst and fractionated by ion exchange chromatography on carboxymethyl cellulose. The derived free nonapeptide, L-arginyl-L-phenylalanyl-L-prolyl-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolyl-L-arginine (XI), retrobradykinin, appeared as the major chromatographic component and behaved as a homogeneous compound on electrophoresis and paper chromatography. Quantitative amino acid analysis of an acid hydrolysate of XI gave the expected values. Another portion of crude Xa was subjected to countercurrent distribution; two hundred transfers in the system *n*-butyl alcohol–acetic acid–water (4:1:5) were sufficient to isolate Xa in analytically pure form. Hydrogenation of pure Xa over a palladium catalyst produced retrobradykinin, XI, in homogeneous form, not requiring further purification.

Experimental

Methyl Nitro-L-argininate Hydrochloride.—Nitro-L-arginine (22 g.) was added to a stirred, cold (−10°) solution of thionyl chloride[8] (14.5 ml.) in methanol (40 ml.). Stirring was continued as the suspension was allowed to warm to room temperature and the resultant solution was refluxed for 2 hr. Repeated evaporation with benzene *in vacuo* produced an oily residue which crystallized readily from methanol–ether, yield 24 g. (84%), m.p. 152–156°; $[\alpha]^{22}D$ +14.5° (*c* 3, methanol), [lit.,[9] m.p. 159–161°; $[\alpha]^{25}D$ +17.5° (*c* 3.2, methanol)].

Carbobenzoxy-L-prolyl-L-proline.—Nitrophenyl carbobenzoxy-L-prolinate (11.1 g.) was added to a solution of L-proline (3.5 g.) in 50% aqueous pyridine (60 ml.). The stirred mixture was maintained at pH 9.2 by the addition of 4 *N* sodium hydroxide. After no further addition of alkali was required, water (80 ml.) was added and the solution was titrated to pH 8 with 4 *N* hydrochloric acid and saturated with sodium bicarbonate. Extraction with an equal volume of ethyl acetate and acidification of the aqueous phase caused the precipitation of the product, which was recrystallized from methanol, yield 7.3 g. (70%), m.p. 187–190°; $[\alpha]^{22}D$ −80.4° (*c* 2, dimethylformamide), [lit.,[10] m.p. 186–187°].

Methyl Carbobenzoxy-L-prolyl-L-prolylnitro-L-argininate (I).—To a solution of carbobenzoxy-L-prolyl-L-proline (10.4 g.) in acetonitrile (50 ml.) containing tri-*n*-butylamine (6.4 ml.) was added, at room temperature, ethyl chloroformate[11] (2.6 ml.). After stirring for 45 min. the resultant mixed anhydride was added to methyl nitro-L-argininate. The latter was prepared from methyl nitro-L-argininate hydrochloride (10 g.) and 1 *N* methanolic sodium methoxide (40 ml.) followed by removal of the methanol by evaporation *in vacuo*. The suspension was stirred at room temperature for 18 hr., the solvent, was removed by evaporation *in vacuo* and the residue was extracted into ethyl acetate. The organic phase was washed with 1 *N* hydrochloric acid, water, saturated sodium bicarbonate and water, dried with anhydrous magnesium sulfate, and evaporated *in vacuo*. The product was dissolved in methanol and precipitated with ether, yield 11.8 g. (76%); $[\alpha]^{20}D$ −86.7° (*c* 2.2, methanol).

Anal. Calcd. for $C_{25}H_{35}N_7O_8$: C, 53.5; H, 6.28; N, 17.5; $-OCH_3$, 5.53. Found: C, 54.2; H, 6.25; N, 17.3; $-OCH_3$, 5.11.

Ethyl Carbobenzoxy-L-phenylalanylglycinate.—To a solution of ethyl glycinate hydrochloride (4.2 g.) in chloroform (30 ml.) containing triethylamine (5.5 ml.) was added nitrophenyl carbobenzoxy-L-phenylalaninate (12.5 g.) and the resultant solution was allowed to stand at room temperature for 48 hr. The solution was diluted with chloroform and extracted with 1 *N* hydrochloric acid, water, 1 *N* ammonium hydroxide, water, and dried over anhydrous magnesium sulfate. The solvent was evaporated *in vacuo* and the residue was recrystallized from ethyl acetate–hexane, yield 9.0 g. (78%), m.p. 106–109°; $[\alpha]^{22}D$ −19.0° (*c* 2, methanol), [lit.,[12] m.p. 110–111°; $[\alpha]^{23}D$ −16.9 (*c* 5, ethanol)].

Anal. Calcd. for $C_{21}H_{24}N_2O_5$: C, 65.6; H, 6.29; N, 7.29. Found: C, 65.9; H, 6.53; N, 7.43.

Ethyl L-Phenylalanylglycinate Hydrobromide (III).—Ethyl carbobenzoxy-L-phenylalanylglycinate (1.2 g.) was dissolved in a saturated solution of hydrobromic acid in acetic acid (8 ml.) and after 1 hr. at room temperature the volatile components were removed by lyophilization. The oily residue was triturated with acetonitrile–ether and then recrystallized from acetonitrile, yield 0.72 g. (88%), m.p. 134–137°; $[\alpha]^{24}D$ +39.2° (*c* 2, water).

Anal. Calcd. for $C_{13}H_{19}N_2O_3Br$: C, 47.1; H, 5.78; N, 8.46; Br, 24.1. Found: C, 47.1; H, 5.90; N, 8.80; Br, 23.9.

Ethyl N-carbobenzoxy-O-acetyl-L-seryl-L-phenylalanylglycinate (IV).—To a solution containing ethyl L-phenylalanylglycinate hydrobromide (III) (3.3 g.) and tri-*n*-butylamine (2.4 ml.) in pyridine (10 ml.), nitrophenyl N-carbobenzoxy-O-acetyl-L-serinate[6] (4 g.) was added, and the solution was left at room temperature for 3 days. Addition of ethyl acetate caused the separation of a solid product which was crystallized from ethanol, yield 3.4 g. (67%), m.p. 172–174° (sinters at 152°); $[\alpha]^{25}D$ −19.5° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{26}H_{31}N_3O_8$. C, 60.8; H, 6.08; N, 8.18. Found: C, 61.6; H, 6.03; N, 8.41.

N-Carbobenzoxy-L-seryl-L-phenylalanylglycine Hydrazide (V).—Ethyl N-carbobenzoxy-O-acetyl-L-seryl-L-phenylalanylglycinate (2 g.) was dissolved in a warm solution of methanol (40 ml.) containing anhydrous hydrazine (95% +) (2 ml.). The solution was allowed to remain at room temperature for 18 hr. during which time the hydrazide separated from solution. After storing in the refrigerator, the product was isolated by filtration and recrystallized from methanol, yield 1.9 g. (94%), m.p. 192–196°; $[\alpha]^{24}D$ −23.1° (*c*, 2, dimethylformamide).

Anal. Calcd. for $C_{22}H_{27}N_5O_6$: C, 57.8; H, 5.95; N, 15.3. Found: C, 57.2; H, 6.37; N, 15.4.

(8) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1114 (1955).

(9) K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).

(10) E. Abderhalden and H. Mienburg, *Fermentforsch.*, **13**, 573 (1933).

(11) T. Wieland and A. Bernhard, *Ann.*, **572**, 190 (1951); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).

(12) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **118**, 405 (1937).

Methyl N-carbobenzoxy-L-seryl-L-phenylalanylglycyl-L-propyl-L-prolylnitro-L-argininate (VI).—Methyl carbobenzoxy-L-prolyl-L-prolylnitro-L-argininate (I) (3.4 g.) was dissolved in acetic acid (3 ml.) and a saturated solution of hydrobromic acid in acetic acid (10 ml.) was added. After 1 hr. at room temperature, ether was added and the solid, hygroscopic methyl L-prolyl-L-prolylnitro-L-argininate hydrobromide (II) was collected by filtration, washed with ether, and dried over potassium hydroxide pellets.

Carbobenzoxy-L-seryl-L-phenylalanylglycine hydrazide (V) (2.48 g.) was dissolved in a mixture of acetic acid (15 ml.) and 1 *N* hydrochloric acid (10 ml.) and the solution was cooled to 0°. Sodium nitrite (0.9 g.) was added and dissolved by shaking and after 10 min. in the cold, the precipitated azide that formed was isolated by filtration and washed thoroughly with ice water. Excess water was pressed out of the filter cake and the azide (Va) was then added to an ice-cold solution of the ester hydrobromide (II) (prepared as described above) in dimethylformamide (10 ml.) containing triethylamine (1.2 ml.). After 48 hr. at 4°, the solvent was removed *in vacuo*, the ensuing oil was dissolved in wet ethyl acetate containing a few milliliters of ethanol and then extracted with 1 *N* hydrochloric acid and water. The organic layer was dried over anhydrous magnesium sulfate and evaporated *in vacuo* and the amorphous residue (VI) was washed with ether, yield 2.6 g. (56%), m.p. sinters at 100° and slowly melts up to 120°; $[\alpha]^{23}D$ −51.2° (*c* 1.2, dimethylformamide).

Anal. Calcd. for $C_{39}H_{52}N_{10}O_{12}$: C, 54.9; H, 6.15; N, 16.4. Found: C, 54.8; H, 6.31; N, 16.3.

Paper chromatography (*n*-butyl alcohol–acetic acid–water 4:1:5) indicated the presence of a single component, ultraviolet absorbing and ninhydrin negative, R_f 0.78.

Carbobenzoxy-L-phenylalanyl-L-proline.—This compound was prepared as described for carbobenzoxy-L-prolyl-L-proline, from nitrophenyl carbobenzoxy-L-phenylalaninate (8.3 g.) and L-proline (2.3 g.). The product was recrystallized from ethyl acetate–hexane, yield 6.5 g. (81%), m.p. 109–111°; $[\alpha]^{22}D$ −37.7° (*c* 2, methanol) [lit.,[13] m.p. 109.5–110.5°]

Anal. Calcd. for $C_{22}H_{24}N_2O_5$: C, 66.7; H, 6.10; N, 7.07. Found: C, 66.2; H, 6.29; N, 6.75.

Carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-proline (VIII).—Carbobenzoxy-L-phenylalanyl-L-proline (2 g.) was dissolved in a saturated solution of hydrobromic acid in acetic acid (3 ml.). After 1 hr., ether was added and the precipitate, L-phenylalanyl-L-proline hydrobromide (R_f 0.54, *n*-butyl alcohol–acetic acid–water 4:1:5, single spot) was washed with ether and dried over potassium hydroxide pellets; yield 1.85 g.

A mixture of carbobenzoxynitro-L-arginine (0.9 g.), triethylamine (0.55 ml.) and N-ethyl-5-phenylisoxazolium-3′-sulfonate[7] (0.95 g.) in dimethylformamide (3 ml.) was stirred at 0° until solution was complete. About 1 hr.was required. To this solution was added a mixture of L-phenylalanyl-L-proline hydrobromide (1.4 g.) as prepared above, in ice-cold dimethylformamide (1.5 ml.) containing triethylamine (0.75 ml.). After stirring at 0° for 1 hr., the suspension was left at room temperature 18 hr. and the solvent was removed *in vacuo*. The oily residue was extracted into ethyl acetate and washed with 1 *N* hydrochloric acid and water. The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed *in vacuo*. The amorphous residue (1.2 g.) crystallized on trituration with ethyl acetate; yield 0.95 g. (61%), m.p. 163–165°; $[\alpha]^{22}D$ −28.1° (*c* 2.2, dimethylformamide) [lit.,[3b] $[\alpha]^{20}D$ −30.2 (*c* 2, dimethylformamide)]. For analysis the compound was recrystallized from acetonitrile.

Anal. Calcd. for $C_{28}H_{35}N_7O_8$: C, 56.3; H, 5.9; N, 16.4. Found: C, 56.4; H, 6.09; N, 16.5.

Nitrophenyl Carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-prolinate (IX).—To a solution of carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-proline (VIII) (0.6 g.) and nitrophenol (0.16 g.) in tetrahydrofuran (2 ml.), dicyclohexylcarbodiimide[14] (0.22 g.) was added. After 24 hr. at room temperature, the suspension was cooled, filtered and the filtrate was evaporated *in vacuo*. The oily residue was triturated with ether–ethanol and recrystallized from methanol; yield 0.5 g. (69%), m.p. 165–167°; $[\alpha]^{23}D$ −33.9° (*c* 2, 1% acetic acid in dimethylformamide).

Anal. Calcd. for $C_{34}H_{38}N_8O_{10}$: C, 56.8; H, 5.33; N, 15.6. Found: C, 56.7; H, 5.18; N, 15.3.

Carbobenzoxynitro-L-arginyl-L-phenylalanyl-L-prolyl-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolylnitro-L-arginine (Xa). — Methyl N-carbobenzoxy-L-seryl-L-phenylalanylglycyl-L-propyl-L-prolylnitro-L-argininate (VI) (1.3 g.) was dissolved in acetic acid (2 ml.) and acetic acid (3 ml.) saturated with hydrobromic acid was added. After 2 hr. at room temperature, ether was added and the precipitated hexapeptide methyl ester hydrobromide (VII) was washed with ether, dried over potassium hydroxide pellets, and dissolved in pyridine (2 ml.) containing triethylamine (0.51 ml.). To this solution of VII was added nitrophenyl carbobenzoxynitro - L - arginyl - L - phenylalanyl - L-prolinate (IX) (0.97 g.) and the mixture was left at room temperature for 48 hr. Ethyl acetate was added to the solution and the precipitate that formed was suspended in 1 *N* sodium hydroxide (6.3 ml.) for 1 hr. at room temperature. Filtration, followed by acidification, produced an oil which solidified on trituration with ethyl acetate, yield 0.68 g. Paper chromatography (*n*-butyl alcohol–acetic acid–water 4:1:5) of the acyl nonapeptide (Xa) revealed two ninhydrin negative, ultraviolet absorbing components. Isolation of pure Xa was achieved by countercurrent distribution (200 transfers) in the system *n*-butyl alcohol–acetic acid–water (4:1:5). Ultraviolet absorption at 270 mμ was employed to follow the course of the distribution. Starting from 0.3 g. of crude X, 0.25 g. of pure material was recovered (over-all yield of pure Xa, 30%) which sintered at 140° and melted up to 155°; $[\alpha]^{22}D$ −47.0° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{58}H_{77}N_{17}O_{17}$: C, 54.2; H, 6.04; N, 18.5. Found: C, 54.9; H, 5.98; N, 18.6.

Retrobradykinin. L-Arginyl-L-phenylalanyl-L-prolyl-L-seryl-L-phenylalanylglycyl-L-prolyl-L-prolyl-L-arginine (XI).—(A). Compound Xa (0.1 g.), unpurified, was hydrogenated over a palladium catalyst for 48 hr. with addition of fresh catalyst after 8 and 24 hr. After filtration from the catalyst and lyophilization, the product was fractionated on a column of carboxymethyl cellulose (4 g.) by gradient elution (0.01 to 0.1 *M* ammonium acetate, pH 6.5) and finally isolated in dry form by lyophilization, yield 40 mg. Paper electrophoresis in ammonium citrate buffer (pH 5.3) or pyridine acetate buffer (pH 4.0) revealed a single component, ninhydrin and Sakaguchi positive, migrating toward the cathode. Compound XI was shown to be homogeneous by paper chromatography. In the system isoamyl alcohol–pyridine–water (3.5:3.5:3) only a single component was present, (R_f 0.14) which was ninhydrin and Sakaguchi positive. After 16 hr. hydrolysis in 6 *N* hydrochloric acid at 110°, quantitative amino acid analysis gave the following amounts (micromoles) of amino acids: arginine, 2.06; glycine, 1.00; phenylalanine, 2.17; proline, 2.93; serine, 0.77. Theoretical ratio: arginine, 2.0; glycine, 1.0; phenylalanine, 2.0; proline, 3.0; serine, 1.0.

(B). Compound Xa, after purification by countercurrent distribution (75 mg.) was hydrogenated as described under (A). The product showed the same degree of purity as that from (A) without additional purification; yield 35 mg.

When tested against the quiescent rat uterus *in vitro*,[3] retrobradykinin (XI) was inactive at the level of 80 γ per

(13) R. E. Neuman, and E. L. Smith, *J. Biol. Chem.*, **193**, 97 (1951).

(14) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

ml. Pre-incubation of the uterus with XI (60 γ per ml.), did not inhibit the typical uterine response to bradykinin (0.02 γ per ml.).

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Syntheses Related to Etiojervane. III. The Synthesis of 1,8-Dimethyl-7-methoxy-1,2,3,4,4a,9a-hexahydrofluoren-2-one[1]

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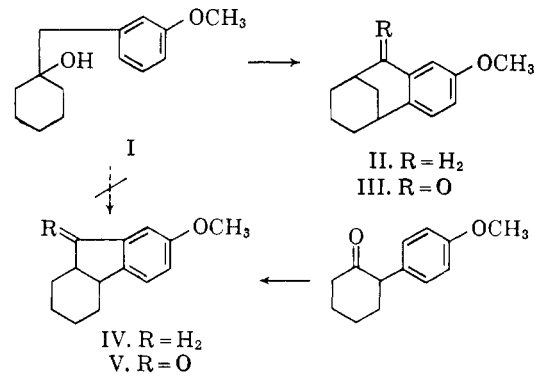
The cyclization of 1-(methoxybenzyl)cyclohexanol (I) did not yield a hexahydrofluorene but rather a substance believed to be 2-methoxy-5,6,7,8,9,10-hexahydro-5,9-methanobenzocycloöctene (II). In an alternate approach the alkylation of Hagemann's ester with benzyl chloride was found to take place at the 2-position. The alkylation products from benzyl chloride and substituted benzyl chlorides could be cyclized either before or after reduction of the double bond to fluorene derivatives. The carboxyl group of 7-methoxy-1,8-dimethyl-1,2,3,4,4a,9a-hexahydrofluorene-2-carboxylic acid (XXIII) was degraded to yield 7-methoxy-1,8-dimethyl-1,2,3,4,4a,9a-hexahydrofluoren-2-one, a ketone which should be readily convertible to a degradation product of jervine.

As indicated in previous publications[3,4] the objective of this investigation has been the preparation of a tricyclic ketone, 1,8-dimethyl-7-methoxy-1,2,3,4,4a,9,9a-hexahydrofluoren-2-one (XXVII) which should be readily convertible to a degradation product of jervine.

In the present work, a first approach was based on the cyclization of a benzylcyclohexanol containing a methoxyl group on the benzene nucleus.[5] Although the cyclization of 1-benzylcyclohexanol has been found[6] to yield 5,6,7,8,9,10-hexahydro-5,9-methanobenzocycloöctene, a consideration of the mechanism suggests that at some time there can be a positive center at C-2 of the cyclohexane ring and it might be possible with a sufficiently active benzene nucleus for a hexahydrofluorene to result. Apparently even with a favorably located methoxyl group, the rate of cyclization was appreciable only with an intermediate having a positive center at C-3. The properties of the cyclization product indicated that alcohol I had behaved exactly as the analogous substance[6] without the methoxyl group. The product (II) could not be dehydrogenated to a fluorene. Furthermore, the 2,4-dinitrophenylhydrazone of ketone III resulting from the chromic acid oxidation was different from the corresponding derivative of ketone V which was prepared by conventional means from 2-(4-methoxyphenyl)cyclohexanone.



A second and successful method started with the alkylation of Hagemann's ester (VI). Previous studies[7] had demonstrated that reaction of VI with an alkyl halide introduced a substituent into the 2-position. To make sure that benzyl halides react in a similar fashion,[8] alkylation product IX was hydrolyzed to ketone XII. The structure of XII was established by aromatization to phenol XIII which had been prepared previously in another way.[9] The alkylations with the substituted benzyl chlorides proceeded readily to yield alkylation products X and XI.

Since XI was less readily accessible, preliminary studies of the cyclization to fluorene derivatives were carried out starting from IX and X. Initial attempts to cyclize the cyclohexene derivative XII directly to a dihydrofluorene were not successful. However, reduction to the cyclohexanone derivative XIV followed by treatment with polyphos-

(1) Presented in part at the 138th National Meeting of the American Chemical Society, New York, N. Y., September 15, 1960.

(2) Abstracted from a thesis presented by M. Sedlak to the Graduate School for the Ph.D. degree, September, 1957.

(3) R. A. Barnes and N. N. Gerber, *J. Org. Chem.*, **26**, 4540 (1961).

(4) R. A. Barnes and R. W. Faessinger, *ibid.*, **26**, 4544 (1961).

(5) Previous studies on the cyclization leading to octahydrophenanthrenes have demonstrated that the products can depend on the reactivity of the benzene ring. See R. A. Barnes, *J. Am. Chem. Soc.*, **75**, 3004 (1953); R. A. Barnes and M. D. Konort, *ibid.*, **75**, 303 (1953).

(6) J. W. Cook and C. L. Hewett, *J. Chem. Soc.*, 62 (1936).

(7) L. I. Smith and G. F. Rouault, *J. Am. Chem. Soc.*, **65**, 631 (1943).

(8) R. Jacquier and S. Boyer, *Bull. soc. chim. France*, **21**, 717 (1954), have previously reported the alkylation of VI with benzyl chloride, but gave no details or proof of structure of the product.

(9) R. C. Huston and A. L. Houk, *J. Am. Chem. Soc.*, **54**, 1506 (1932).