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Synthesis of a Biologically Active Analog of Lysine-vasopressin, with Phenylalanine Replacing Tyrosine: 2-Phenylalanine Lysine-vasopressin[1]

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RECEIVED JUNE 30, 1960

2-Phenylalanine lysine-vasopressin is an analog of lysine-vasopressin in which the tyrosyl residue of the hormone is replaced by the phenylalanyl grouping. This analog has been synthesized through the protected nonapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-phenylalanyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-Nϵ-tosyl-L-lysylglycinamide, which was itself prepared by the successive attachment of L-phenylalanine and S-benzyl-N-carbobenzoxy-L-cysteine to the appropriate heptapeptide amide with the use of the nitrophenyl ester method. Conversion of the nonapeptide intermediate to 2-phenylalanine lysine-vasopressin was effected by treatment with sodium in liquid ammonia followed by oxidation in air. After purification by ion-exchange chromatography, the 2-phenylalanine lysine-vasopressin possessed approximately one-fifth of the pressor activity exhibited by lysine-vasopressin. Thus, the presence of the phenolic hydroxyl group in lysine-vasopressin enhances the pressor activity of the hormone but is not essential for the possession of this activity.

In connection with studies on the relation of structure to the biological activity of vasopressin, an analog of lysine-vasopressin has been synthesized in which the tyrosyl residue is replaced by the phenylalanyl residue.[2] Earlier studies on synthetic 2-phenylalanine oxytocin[3,4] had shown this analog to possess the biological activities characteristic of oxytocin although they occurred to a smaller degree in the analog than in the hormone. It was thus evident that the presence of the phenolic hydroxyl group contributed to the biological activities of oxytocin but was not essential for them. The same has now been found to be the case with 2-phenylalanine lysine-vasopressin (I).

H-CyS-Phe-Phe-Glu(NH_2)-Asp(NH_2)-CyS-Pro-Lys-Gly-NH_2

(1) (2) (3) (4) (5) (6) (7) (8) (9)

2-Phenylalanine lysine-vasopressin

The synthesis of 2-phenylalanine lysine-vasopressin was accomplished by use of the nitrophenyl ester method[5] in a series of reactions similar to those used recently for the synthesis of lysine-vasopressin.[6] In fact, the crystalline heptapeptide amide intermediate for lysine-vasopressin, namely carbobenzoxy-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-Nϵ-tosyl-L-lysylglycinamide (II),[6] served as starting material for 2-phenylalanine lysine-vasopressin. Following removal of the carbobenzoxy group with HBr in acetic acid, reaction with the *p*-nitrophenyl ester of carbobenzoxy-L-phenylalanine, removal of the carbobenzoxy group from the resulting protected octapeptide and treatment with the *p*-nitrophenyl ester of S-benzyl-N-carbobenzoxy-L-cysteine led to an excellent yield of the protected nonapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-phenylalanyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-Nϵ-tosyl-L-lysylglycinamide (III).

Cleavage of the protecting groups from III by treatment with sodium in liquid ammonia, ring closure through formation of the disulfide bond of cystine by oxidation with air and isolation of the 2-phenylalanine lysine-vasopressin after desalting on an ion-exchange column were carried out according to the procedures already described for lysine-vasopressin.[7] The product thus obtained possessed 43 pressor units/mg. Purification was carried out by ion-exchange chromatography on Amberlite IRC-50 (XE-64)[8] or by electrophoresis on a cellulose supporting medium.[9] The 2-phenylalanine lysine-vasopressin appeared to be homogeneous on ion-exchange chromatography, paper chromatography and paper electrophoresis. Chromatography on the starch column and elementary analysis also gave the expected results. A highly purified preparation showed, per mg., 50–60 units of rat pressor activity,[10] approximately 0.5 unit of avian depressor activity[11] and less than 0.1 unit of rat uterine-contracting activity.[12] These values are in general agreement with those reported by Boissonnas and Guttmann.[2]

Experimental[13]

Carbobenzoxy-L-phenylalanyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-Nϵ-tosyl-

(1) This work was supported in part by a grant from the National Heart Institute, Public Health Service, Grant H-1675. One of the authors (J. M.) is indebted to the Conference Board of Associated Research Councils (Washington) and to the Fulbright Commission in Germany (Bad Godesberg) for a Fulbright Travel Grant.

(2) After this work had been completed and the manuscript was being prepared for publication, a paper appeared by R. A. Boissonnas and St. Guttmann [*Helv. Chim. Acta*, **43**, 190 (1960)] in which the synthesis of 2-phenylalanine lysine-vasopressin and the pharmacological effects of the compound were described. They prepared the protected nonapeptide by the coupling of the azide of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-phenylalanyl-L-phenylalanine with L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-Nϵ-tosyl-L-lysylglycinamide. We have proceeded with the presentation of our work, since the methods used for the synthesis of the protected nonapeptide intermediate in the two Laboratories differed considerably.

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(13) Capillary melting points were determined for all compounds and are corrected.

L-lysylglycinamide (III).—To a solution of II (2.34 g.) in acetic acid (10 ml.) 3 *N* HBr in acetic acid (20 ml.) was added. After 1 hr. at room temperature dry ether (250 ml.) was added. The precipitate was filtered, washed thoroughly with ether and dried *in vacuo* over KOH and $CaCl_2$. The hydrobromide was then dissolved in dimethylformamide (10 ml.) and triethylamine (1.6 ml.) was added with cooling. *p*-Nitrophenyl carbobenzoxy-L-phenylalaninate[3] (840 mg.) was added to the reaction mixture. After 45 hr. at 35° the almost solid mass was triturated with ethyl acetate (100 ml.), filtered and washed successively with ethyl acetate, ethanol and ethyl acetate; yield 2.56 g., m.p. 222° dec. After reprecipitation of the material from dimethylformamide by the addition of ethanol, the melting point was unchanged; $[\alpha]^{23}D$ −42.3° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{65}H_{79}O_{14}N_{12}S_2$: C, 59.3; H, 6.05; N, 12.8; S, 4.87. Found: C, 59.1; H, 6.12; N, 12.6; S, 4.78.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-phenylalanyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide (IV).—The free base, prepared from III (1.98 g.) by treatment with 2 *N* HBr in acetic acid, was allowed to react with *p*-nitrophenyl S-benzyl-N-carbobenzoxy-L-cysteinate[14] (840 mg.). The product was isolated in the manner described in the preceding section and purified by precipitation from a dimethylformamide solution with ethyl acetate; wt. 1.9 g. (88%), m.p. 222–223°. After reprecipitation of the compound from acetic acid–ethanol the melting point remained unchanged; $[\alpha]^{21}D$ −43.0° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{75}H_{91}O_{15}N_{13}S_3$: C, 59.6; H, 6.08; N, 12.0; S, 6.37. Found: C, 59.3; H, 6.05; N, 12.0; S, 6.25.

2-Phenylalanine Lysine-vasopressin (I).—The protected nonapeptide IV (500 mg.) was dissolved in liquid ammonia (600 ml.) which had been distilled from sodium. Sodium was added until a blue color persisted for 3 min. (approximately 95 mg. of sodium). Acetic acid (0.5 ml.) was added and the ammonia was then evaporated to a small volume (20 ml.). The rest of the ammonia was evaporated from the frozen state on a water pump with a KOH drying jar between flask and pump. The residue was washed with freshly distilled ethyl acetate (200 ml.) and then dissolved in 1 l. of oxygen-free redistilled water at approximately 0°. The *p*H of the solution was 6.5–6.8. Air was passed through the solution for 3 hr., when assay for rat pressor activity showed a total of 18,000 units. The *p*H was adjusted with acetic acid to 4.0, and the solution was passed through an Amberlite IRC-50 (XE-64) column (1.9 × 12.5 cm.) in the H^+ form for desalting.[15] The column was washed with 0.25% acetic acid (450 ml.) and water (20 ml.), and the product was then eluted with a 30% pyridine–4% acetic acid solution. After lyophilization of the eluate (about 20 ml.) 380 mg. of material with a pressor activity of 43 units/mg. was obtained, representing a total pressor activity of approximately 16,000 units.

The product was submitted to ion-exchange chromatography essentially according to the procedure already described for the purification of lysine-vasopressin.[7,8] The material (250 mg., containing approximately 11,000 pressor units) was dissolved in 1 ml. of 0.5 *M* ammonium acetate buffer of *p*H 6.38 (20°) and placed on an Amberlite IRC-50 (XE-64) column (1.9 × 43.5 cm.) which had been equilibrated with the same buffer. The chromatogram was developed with the same buffer at room temperature with a flow rate of 5 ml. per hr. and 85 fractions were collected. The volume per fraction was 2.8 ml. The fractions were analyzed by determination of the absorption at 275 mμ, Folin–Lowry color reaction[16] and pressor activity. A small inactive peak (Tubes 20–30) was separated from the main peak (Tubes 47–70) which contained the activity. The recovery in terms of activity in the main peak was 83% and the recovery in terms of weight, 64%. Tubes 47–49, 50–55, 56–60 and 61–70 were pooled, aliquots removed for pressor assay and the solutions lyophilized three times to remove the ammonium acetate. The results are summarized in Table I. The most active fractions of 2-phenylalanine lysine-vasopressin exhibited a pressor activity of 56–60 units/mg. and an avian depressor activity of 0.5 unit/mg. This analog showed less than 0.1 unit/mg. of activity in the rat uterine strip assay.

TABLE I

ION-EXCHANGE CHROMATOGRAPHY OF 2-PHENYLALANINE LYSINE-VASOPRESSIN

Fraction	Activity before lyophil.	Wt. after lyophil., mg.	Pressor activity after lyophil., units/mg.	Total pressor activity in fraction
47–49	750	13	48	630
50–55	4000	69	60	4200
56–60	2300	41	56	2300
61–70	2200	38	49	1900
Recovery		161 mg.		9030

A sample of the crude 2-phenylalanine lysine-vasopressin (120 mg. containing approximately 4800 pressor units) was also purified by electrophoresis on powdered cellulose.[9] The material was dissolved in 0.7 ml. of 0.1 *M* pyridine-acetate buffer of *p*H 4.0 and placed 6 cm. from the anode end of a cellulose block[17] (46 × 10 × 1 cm.). A current of 400 volts and 34–40 milliamperes was applied at 0° for 50 hr. The 6-cm. main band had traveled 25.8 cm. Some trailing material was found to be inactive. The block was cut into segments (1.25 cm.) and the solution pressed from each segment. The pressor activity, Folin–Lowry color reaction and weight were determined for each segment. The material in the three segments representing the main band (52 mg.) possessed a pressor activity of 50–58 units/mg.

Paper electrophoresis on Whatman No. 3 MM with 0.1 *M* pyridine–acetate buffer of *p*H 4.0 with 400 volts showed the purified 2-phenylalanine lysine-vasopressin to travel as a single spot at the same rate as lysine-vasopressin. Paper chromatography on Whatman No. 1 paper with the solvent system butanol–acetic acid–water (4:1:5) showed a single spot with an R_f value compared to lysine-vasopressin (1) of 1.36. Chromatography of an hydrolysate of 2-phenylalanine lysine-vasopressin on the starch column[18] showed the amino acids and ammonia to be present in the following molar ratios, with the ratio for glycine arbitrarily taken as 1: phenylalanine 1.9, proline 0.7, glutamic acid 1.0, aspartic acid 1.0, glycine 1.0, lysine 1.0, cystine 1.0 and ammonia 3.1. A sample for elementary analysis was dried at 100° for 8 hr. *in vacuo* over P_2O_5.

Anal. Calcd. for $C_{48}H_{65}O_{11}N_{13}S_2 \cdot C_2H_4O_2$: C, 52.4; H, 6.32; N, 16.5. Found: C, 52.9; H, 6.31; N, 16.9.

Acknowledgments.—The authors wish to thank Mr. Joseph Albert for the microanalyses reported herein, Miss Maureen O'Connell and Miss Dade Tull for the biological assays and Mrs. Lorraine S Abrash for the starch column chromatography.

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