The Structure of Acetone-lysine-vasopressin as Established through Its Synthesis from the Acetone Derivative of S-Benzyl-L-cysteinyl-L-tyrosine[1]

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Abstract: Acetone-lysine-vasopressin, an isopropylidene derivative resulting from the inactivation of the hormone by acetone, has been synthesized from the acetone derivative of S-benzyl-L-cysteinyl-L-tyrosine. This isopropylidene dipeptide was first coupled with L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide by the mixed anhydride procedure. The resulting nonapeptide derivative was then converted to acetone-lysine-vasopressin by removal of the tosyl and the benzyl groups with sodium in liquid ammonia and subsequent oxidation of the resulting disulfhydryl compound with potassium ferricyanide. Isolation and purification of the synthetic acetone-lysine-vasopressin was accomplished by partition chromatography on Sephadex. Extensive comparison of the physical and chemical properties, as well as the pressor activity (6–7 units/mg), of the synthetic material with those of acetone-lysine-vasopressin prepared from lysine-vasopressin and acetone showed the two compounds to be identical. Since the acetone derivative of S-benzyl-L-cysteinyl-L-tyrosine has been shown previously to be a substituted 2,2-dimethyl-4-imidazolidinone, it may be concluded that acetone-lysine-vasopressin possesses the same imidazolidinone ring structure in which the isopropylidene group from acetone forms a bridge between the nitrogen atom of the free amino group of the half-cystine residue at position 1 in lysine-vasopressin and the nitrogen of the peptide bond between this half-cystine residue and the tyrosine residue at position 2.

The inactivation of lysine-vasopressin (Figure 1) by acetone with the formation of acetone-lysine-vasopressin and reconversion of the latter compound to the hormone have recently been reported.[3] The analytical data obtained on this compound were in agreement with the elemental composition calculated for a monoisopropylidene derivative of lysine-vasopressin. Involvement of the amino group on the half-cystine residue at position 1 of lysine-vasopressin in the formation of the derivative was indicated by the fact that no change in pressor potency was observed upon treatment of 1-deamino-8-lysine-vasopressin[4] with acetone under the same conditions.[3] These results on acetone-lysine-vasopressin parallel those obtained on acetone-oxytocin.[5]

Recently Yamashiro and du Vigneaud[6] have demonstrated that a product identical with acetone-oxytocin is obtained by condensation of an isopropylidene derivative of S-benzyl-L-cysteinyl-L-tyrosine with the heptapeptide L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, followed by removal of the benzyl groups with sodium in liquid ammonia[7] and subsequent oxidation of the resulting disulfhydryl intermediate. The present paper describes a comparable synthesis of acetone-lysine-vasopressin starting with isopropylidene-S-benzyl-L-cysteinyl-L-tyrosine and the appropriate heptapeptide.

The isopropylidene dipeptide derivative was treated with isobutyl chlorocarbonate[8] and then coupled with L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide.[9] The resulting product, presumably the isopropylidene derivative of S,S′-dibenzyl-lysine-vasopresseine, was then treated with sodium in liquid ammonia for removal of the tosyl and S-benzyl groups, and the disulfhydryl intermediate so obtained was titrated in neutral aqueous solution with potassium ferricyanide until the oxidation was complete. The resulting solution was lyophilized, and the crude product was subjected to partition chromatography on Sephadex G-25 at 25° in 1-butanol–pyridine–0.2 *N* acetic acid (6:1:7). Analysis of the eluates by the Folin–Lowry method[10] gave a chromatogram containing a major peak with an R_f of 0.15. An authentic sample of acetone-lysine-vasopressin (prepared from acetone and lysine-vasopressin) exhibited an R_f value of 0.16 upon chromatography under the same conditions. The material represented by the major peak (R_f 0.15) was isolated and rechromatographed on Sephadex G-25 at 4° in 1-butanol–ethanol–pyridine–0.2 *N* acetic acid (14:2:5:24) according to the procedure described previously for the isolation of acetone-lysine-vasopressin.[3] One peak was detected with R_f 0.36, which is the R_f value of an authentic sample of acetone-lysine-vasopressin when chromatographed under the same conditions.

The synthetic material liberates acetone when it is

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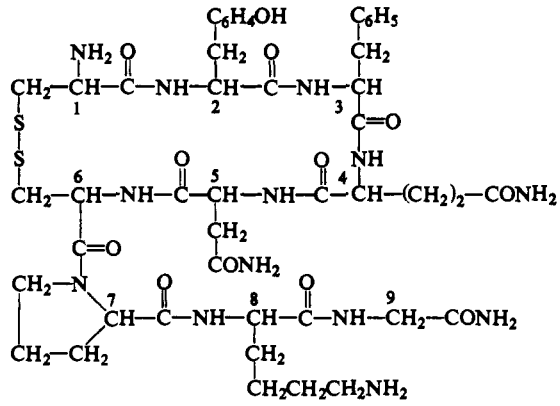

Figure 1. Structure of lysine-vasopressin with numbers indicating the position of the individual amino acid residues.

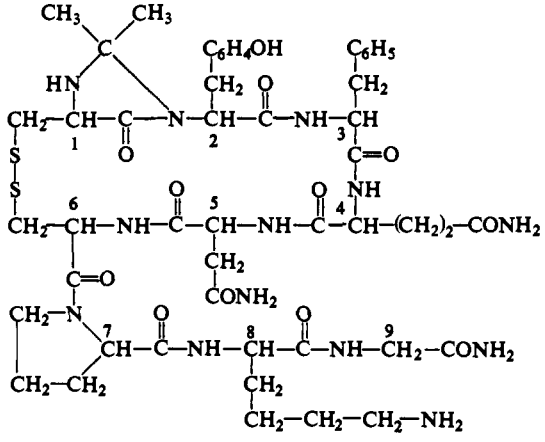

Figure 2. Structure of acetone-lysine-vasopressin with numbers indicating the position of the individual amino acid residues.

heated in aqueous solution at 90° for 15 min, as was also observed with acetone-lysine-vasopressin.[3] When a sample of the material is boiled in 0.1 *N* acetic acid for 15 min with simultaneous distillation, a quantitative determination of acetone in the distillate shows that the amount present is the same as that released by acetone-lysine-vasopressin under similar conditions.

The synthetic material and authentic acetone-lysine-vasopressin show identical behavior not only on partition chromatography on Sephadex G-25 in the two different solvent systems at two different temperatures, as already described, but also when they are subjected to gel filtration on Sephadex G-25 in 0.2 *N* acetic acid. The synthetic and authentic materials migrate at the same rate on electrophoresis at pH 5.6, and their optical rotations in 0.1 *N* acetic acid in 67% aqueous acetone are in agreement with each other. The synthetic material exhibits the same low rat pressor activity (6–7 units/mg) that was observed for acetone-lysine-vasopressin.[3]

These comparisons demonstrate that the product obtained by synthesis starting from the isopropylidene derivative of S-benzyl-L-cysteinyl-L-tyrosine is identical with acetone-lysine-vasopressin obtained by the reaction of lysine-vasopressin with acetone.

In a recent communication,[11] convincing evidence was presented that the isopropylidene derivative of S-benzyl-L-cysteinyl-L-tyrosine formed by treatment of the dipeptide with acetone is a substituted 2,2-dimethyl-4-imidazolidinone. Since acetone-oxytocin was synthesized from this imidazolidinone, it was concluded that acetone-oxytocin possesses the same imidazolidinone ring structure in which the isopropylidene group from acetone forms a bridge between the nitrogen atom of the free amino group of the half-cystine residue at position 1 in oxytocin and the nitrogen of the peptide bond between this half-cystine residue and the tyrosine residue at position 2. Since acetone-lysine-vasopressin has now been synthesized from the same substituted 2,2-dimethyl-4-imidazolidinone, it may be concluded that acetone-lysine-vasopressin possesses the structure shown in Figure 2, which is analogous to that of acetone-oxytocin.

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Experimental Section

Methods. Sephadex G-25 partition columns were prepared and operated as described previously.[12] Gel filtration was performed on Sephadex G-25 block polymerizate (200–270 mesh). Peptide materials in the column effluents were detected by the Folin–Lowry method.[10]

For qualitative detection of acetone by a modified Legal test,[13] solutions were spotted on "Acetest" reagent tablets (Ames Co., Inc., Elkhart, Ind.). A test was considered positive when a permanent purple color developed similar to that given by 0.1% aqueous acetone.

For the quantitative determination of acetone liberated by the isopropylidene derivative, a sample (1 mg) was heated in 5 ml of 0.1 *N* acetic acid in an apparatus where the distillate could be collected at 0° in a 5-ml volumetric flask containing 2 ml of water. The sample solution was heated near the boiling point for 5 min and then was boiled vigorously until almost 3 ml of distillate had been collected. Appropriate aliquots of the distillate were analyzed by the salicylaldehyde method.[14]

Paper electrophoresis was carried out at 4° in pyridine acetate buffer at pH 5.6 at 300 V for 18 hr, and the Pauly reagent was used for color development. Rat pressor activity[15] was measured against the USP posterior pituitary reference standard.

Acetone-lysine-vasopressin (Isopropylidene-lysine-vasopressin). Isopropylidene-S-benzyl-L-cysteinyl-L-tyrosine (169.6 mg) was dissolved in 2.37 ml of dry tetrahydrofuran and cooled to −25° before the addition of 0.056 ml of triethylamine, followed by 0.052 ml of isobutyl chlorocarbonate. The mixture was warmed to −10° over a 15-min period and then cooled to −40°. A solution of 399 mg of the hydrobromide of L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide[9] in 3.0 ml of dimethylformamide was added, and the mixture was titrated to pH 7.5 with cold triethylamine and then gradually warmed to 15° over a period of 5 hr. The thick mixture was diluted with 36 ml of ice-cold water and stirred overnight at 4°. The crude product was collected on a filter at 4°, washed with two 5-ml portions of ice-cold water, and dried *in vacuo* over P_2O_5 at 4°, yielding 401 mg.

This crude protected nonapeptide was dissolved in 125 ml of liquid ammonia (distilled from sodium) and treated with sodium until a blue color persisted throughout the solution for 1 min. The solution was evaporated *in vacuo* to a low volume and lyophilized. The resulting powder was dissolved in 900 ml of deaerated water containing 0.2 ml of trifluoroacetic acid, and the pH of the solution was adjusted to 8.0 with a few drops of 2 *N* NH_4OH. The solution was treated with 57 ml of 0.01 *N* potassium

(12) D. Yamashiro, *Nature*, **201**, 76 (1964); D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **88**, 1310 (1966).

(13) The test, used clinically for detection of ketone bodies, is described in K. Diem, Ed., "Documenta Geigy: Scientific Tables," 6th ed, Geigy Pharmaceuticals, Ardsley, N. Y., 1962, pp 532, 537.

(14) J. A. Behre and S. R. Benedict, *J. Biol. Chem.*, **70**, 487 (1926); R. N. Harger and R. B. Forney, "Standard Methods of Chemical Analysis," Vol. 2, F. J. Welcher, Ed., D. Van Nostrand Co., Inc., Princeton, N. J., 1963, p 2139.

(15) "The Pharmacopeia of the United States of America," 17th rev, Mack Publishing Co., Easton, Pa., 1965, p 749.

ferricyanide while 1 N NH_4OH was added to maintain a pH of 7. An excess of 0.01 N potassium ferricyanide solution (5 ml) was then added and a yellow color persisted for at least 20 min. The solution was stirred with 35 ml (wet volume) of the chloride form of AG3X4 resin (Bio-Rad Laboratorties, Richmond, Calif.) for 15 min to remove ferrocyanide and excess ferricyanide ions. The oxidation and ion-exchange procedures were carried out within a period of 1 hr at 25°. The solution obtained after removal of the resin by filtration was lyophilized.

The crude product was subjected to partition chromatography on Sephadex G-25 at 25° under the following conditions: solvent, 1-butanol–pyridine–0.2 N acetic acid (6:1:7); column size, 2.85 × 58.0 cm; hold-up volume, 78.7 ml; flow rate, 25 ml/hr; fraction size, 9.2 ml; aliquots for Folin–Lowry determinations, 0.10 ml; solvent for regeneration of column, pyridine–0.1% acetic acid (1:4). One major peak was detected in the chromatogram with an R_f value of 0.15. Partition chromatography of an authentic sample (2 mg) of acetone-lysine-vasopressin under the same conditions gave a peak with R_f 0.16. The eluates represented by the peak with R_f 0.15 were mixed with 360 ml of ice-cold water, evaporated *in vacuo* to low volume in a bath at 15°, and lyophilized, yield 58 mg. This product was rechromatographed on Sephadex G-25 at 4° under the following conditions: solvent, 1-butanol–ethanol–pyridine–0.2 N acetic acid (14:2:5:24); column size, 2.20 × 52 cm; hold-up volume, 49.3 ml; flow rate, 12 ml/hr; fraction size, 3.3 ml; aliquots for Folin–Lowry determination, 0.05 ml; solvent for regeneration of column, pyridine–0.1% acetic acid (1:4). One peak was detected with R_f 0.36, and the material isolated from the eluates represented by the central portion of the peak weighed 42 mg. Chromatography of an authentic sample of acetone-lysine-vasopressin under the same conditions gave a peak with R_f 0.36.

Comparisons of Synthetic and Authentic Acetone-lysine-vasopressin. A sample (1 mg) of the synthetic material dissolved in 0.05 ml of 0.1% acetic acid gave a negative Legal test for acetone, but after the solution was heated at 90° for 15 min a positive test was obtained. A quantitative determination showed that 89% of the theoretical amount of acetone had been liberated from the compound. The results in these two tests were the same, within experimental error, as those obtained with an authentic sample of acetone-lysine-vasopressin.[3] The synthetic preparation (1.2 mg) was subjected to gel filtration on Sephadex G-25 as follows: solvent, 0.2 N acetic acid at 25°; column size, 1.38 × 83.5 cm; flow rate, 7 ml/hr; fraction size, 0.79 ml; aliquots for Folin–Lowry determination, 0.79 ml. Only one peak, with a maximum at effluent volume 102 ml, was detected. This is the same effluent volume at which the maximum occurred on gel filtration of authentic acetone-lysine-vasopressin.

The synthetic material exhibited the specific rotation $[\alpha]^{21}D$ −72.4° (*c* 0.5, 0.1 N acetic acid in 67% aqueous acetone), as compared to $[\alpha]^{17}D$ −74° (*c* 0.5, 0.1 N acetic acid in 67% aqueous acetone) reported for acetone-lysine-vasopressin.[3] The synthetic material has a pressor activity of 6–7 units/mg, the same as that previously reported for acetone-lysine-vasopressin.[3]

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Biosynthesis of the *Cinchona* Alkaloids. II. The Incorporation of Tryptophan-1-^{15}N,2-^{14}C and Geraniol-3-^{14}C into Quinine[1]

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Abstract: The administration of DL-tryptophan-1-^{15}N,2-^{14}C (prepared from indole-1-^{15}N,2-^{14}C) to *Cinchona succirubra* plants yielded labeled quinine having all its ^{14}C at C-9, and excess ^{15}N only on the quinoline nitrogen. Furthermore, the specific incorporation of ^{14}C and ^{15}N into these positions was identical (0.97%). These results strongly support the hypothesis of Goutarel, Janot, Prelog, and Taylor, who suggested in 1950 that quinine and related quinoline alkaloids found in *Cinchona* species are derived from indole alkaloids of the corynantheine type. Additional evidence favoring this biosynthetic scheme was obtained by feeding geraniol-3-^{14}C to the same species, when quinine labeled specifically at C-10 of the quinuclidine nucleus was obtained. This result is in agreement with recent work on the origin of the nontryptophan-derived portion of indole alkaloids containing an extra nine- or ten-carbon unit.

We have previously shown[3] that the administration of tryptophan-α-^{14}C (label in the alanyl side chain) to *Cinchona succirubra* plants yielded quinine (**9**) which had essentially all its activity located at C-2′ of its quinoline moiety, This result was in accord with the biosynthetic scheme illustrated in Scheme I, which is an elaboration of the one suggested by Goutarel, *et al.*,[4] in 1950. It was proposed that quinine and related *Cinchona* alkaloids are formed from an indole derivative such as **6**, which is plausibly formed by a Mannich reaction between tryptophan (**1**) and the ten-carbon unit **2**, the origin of which will be discussed later. The alkaloid corynantheine has the carbon skeleton of compound **6**. Cleavage of the tetrahydro-β-carboline ring, loss of the carboxyl group, and formation of the quinu-

(1) This investigation was supported by Research Grant GM-13246 from the U. S. Public Health Service. A preliminary account of part of this work has appeared as a communication: E. Leete and J. N. Wemple, *J. Am. Chem. Soc.*, **88**, 4743 (1966).

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