Synthesis and Biological Properties of 1-Acetyl-8-lysine-vasopressin*

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Our interest in acetyl derivatives of the posterior pituitary hormones was stimulated by the papers of Sonenberg and Money et al. (1-3) on their studies with acetylated thyrotropin. They found that preparations of the thyrotropic hormone, after being treated with acetic anhydride, exhibit no thyroid-stimulating activity but exert an inhibitory action toward the thyrotropic hormone. We performed experiments similar to theirs in which we treated oxytocin and 8-arginine-vasopressin with acetic anhydride. The crude product from oxytocin was devoid of avian depressor activity, but was capable of inhibiting to some extent the avian depressor effect of oxytocin. The crude product from 8-arginine-vasopressin exhibited no pressor activity, but caused a reduction in the normal pressor response to 8-arginine-vasopressin in the rat. Later, we synthesized 1-acetyl-8-arginine-vasopressin, the derivative of 8-arginine-vasopressin in which the N-terminal amino group is acetylated, and studied its effects in the rat pressor assay (5). Like the earlier crude acetylation product, 1-acetyl-8-arginine-vasopressin was devoid of pressor activity, but was capable of inhibiting the pressor effect of 8-arginine-vasopressin. As part of a continuation of this study, we have synthesized the corresponding derivative of 8-lysine-vasopressin, 1-acetyl-8-lysine-vasopressin (1-(N-acetyl-hemis Cys) 8 lysine vasopressin) (Fig. 1). The present paper describes the synthesis of this peptide and presents the results of a more detailed study of its biological properties.

The synthesis was accomplished by way of the protected nonapeptide N-acetyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-phenylalaninyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N'-tosyl-L-lysylglycinamide. The starting material was the protected octapeptide O-benzyl-N-carboxbenzoxyl-L-tyrosyl-L-phenylalaninyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N'-tosyl-L-lysylglycinamide, an intermediate in the synthesis of 8-lysine-vasopressin by the p-nitrophenyl ester method (6). The protected octapeptide was treated with hydrogen bromide in glacial acetic acid in order to remove the blocking groups from the tyrosine residue. The liberated octapeptide amide was allowed to react in the presence of triethylamine with p-nitrophenyl N-acetyl-S-benzyl-L-cysteinamide to yield the protected nonapeptide.1-Acetyl-8-lysine-vasopressin was prepared from the protected nonapeptide by first removing the tosyl group from the lysine residue and the benzyl groups from the half-cystine residues with sodium in liquid ammonia (8), and then converting the resulting sulfhydryl peptide into the cyclic disulfide with the aid of potassium ferricyanide (9). The crude product was purified by ion exchange chromatography and countercurrent distribution. The purified material appeared to be homogeneous when subjected to paper chromatographic and paper electrophoretic analysis. Elementary analysis and analysis for amino acids and ammonia gave the expected results for 1-acetyl-8-lysine-vasopressin.

The biological activity of the chromatographically purified 1-acetyl-8-lysine-vasopressin was studied under the conditions of various bioassays that are used in the study of posterior pituitary hormones. Both the ability to exert the typical biological effects of the vasopressins and oxytocin, and the ability to inhibit these effects were studied.

Under the conditions of the assay for pressor activity in the rat (10), 1-acetyl-8-lysine-vasopressin did not increase the blood pressure when injected in amounts up to 100 μg. However, injections of the acetylated hormone reduced the response to simultaneous or subsequent injections of the U.S.P. Posterior Pituitary Reference Standard. In a typical experiment, 1 minute after a dose of 100 μg of 1-acetyl-8-lysine-vasopressin the pressor response to 5 milliunits of the standard was reduced to about 15% of the normal response. The normal response to an injection of standard was not regained until approximately 45 minutes had elapsed.

In the avian depressor assay (11), 1-acetyl-8-lysine-vasopressin did not affect the blood pressure when injected in amounts up to 600 μg. The effect of the U.S.P. standard in this assay also was inhibited by previous or simultaneous injections of the acetylated hormone. The decrease in blood pressure produced by 30 milliunits of standard could be prevented completely by the simultaneous injection of 100 μg of 1-acetyl-8-lysine-vasopressin. Inhibition remained in effect for 10 to 25 minutes.

1-Acetyl-8-lysine-vasopressin exerted a low but detectable activity when assayed for inhibition of diuresis in the hydrated rat (12, 13), for contraction of the isolated rat uterus in a bath free from magnesium ions (14, 15), for elevation of mammary gland pressure in the lactating rabbit (16, 17), and for contraction and the results of a study of its ability to form a peptide without the occurrence of detectable racemization have been reported elsewhere (7).

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"W. D. Cash and R. O. Studer, unpublished experiments, referred to in an earlier paper from this laboratory (4).
The synthesis of p-nitrophenyl N-acetyl-S-benzyl-L-cysteinamide has been reported (7).
The acetylation of the NH₂-terminal amino group with an acetyl substituent leads to almost complete biological inactivation of both 8-lysine-vasopressin and oxytocin (21). Studies in other laboratories have indicated that acetylation leads to comparable reductions in the biological activities of the posterior pituitary hormones. An acetyl derivative of 8-lysine-vasopressin prepared by treatment of the hormone with acetic anhydride in glacial acetic acid, and purified by ion exchange chromatography in the rat an anti-diuretic activity equivalent to approximately 0.05% of the antidiuretic activity of 8-lysine-vasopressin (19). Bis-(acetyl)-lysine-vasopressin, prepared by the treatment of 8-lysine-vasopressin with acetic anhydride in the presence of triethylamine and purified by countercurrent distribution, exhibited a pressor activity equivalent to less than 0.4% of the pressor activity of 8-lysine-vasopressin (20). N-Acetyl-oxytocin, the preparation and purification of which were not described, was devoid of avian depressor activity, but exerted oxytocic and milk-ejecting activities equivalent to about 0.4% of the corresponding activities of oxytocin (21).

The fact that the blocking of the NH₂-terminal amino group with an acetyl substituent leads to almost complete biological inactivation of both 8-lysine-vasopressin and oxytocin cannot be interpreted as an indication that the free amino group of either hormone is essential for biological activity. Derivatives of oxytocin and 8-lysine-vasopressin in which the NH₂-terminal amino groups are replaced by hydrogen atoms exhibit to a high degree all of the typical biological activities of the posterior pituitary hormones (9, 22, 23). In fact, some of the activities of desamino-oxytocin and 1-desamino-8-lysine-vasopressin are higher than the corresponding activities of the unmodified hormones.

The reason for the marked change in biological activity attributable to the presence of the acetyl group remains to be established. Clues may be provided by further chemical and biological studies with acetyl derivatives, and perhaps with other acyl derivatives of the vasopressins and oxytocin.

It is noteworthy that inhibitory activity was exhibited by 1-acetyl-8-lysine-vasopressin only in those assays in which changes in blood pressure were measured. Similarly, the only assay in which N-acetyl-oxytocin was reported to exert an inhibitory effect was the avian depressor assay (21). In this connection, recent findings suggest that the receptor sites on blood vessels for the pressor action of the vasopressins are different from the receptors for the vasopressins and oxytocin on uteri and myoepithelial cells (24).

The reported observation that the presence of an acetyl substituent on the NH₂-terminal amino group of certain peptides enhances their melanocyte-stimulating activity (25) prompted us to test 1-acetyl-8-lysine-vasopressin for this activity. The assay based upon the darkening of Anolis carolinensis skin in vitro (20) was employed. Pieces of Anolis skin did not darken when placed in Ringer's solution that contained either synthetic oxytocin, lysine-vasotocin (28), or histidine-vasopressin (29). The skin darkened readily in Ringer's solution that contained an extract of beef posterior pituitary powder (27). A few other related peptides were tested for melanocyte-stimulating activity. Anolis skin did not darken when placed in Ringer's solution that contained up to 100 µg per ml of either synthetic oxytocin, lysine-vasotocin (28), or histidine-vasopressin (29). The skin darkened readily in Ringer's solution that contained 0.25 µg per ml of several different samples of highly purified 8-lysine-vasopressin that had been isolated from natural sources, the activity probably being attributable to traces of melanophore-stimulating hormone present in the samples.

Fig. 1. 1-Acetyl-8-lysine-vasopressin darkened readily in Ringer's solution that contained an extract of beef posterior pituitary powder (27).
**1-Acetyl-8-lysine-vasopressin** — Protected nonapeptide I, 100 mg, was dissolved in 70 ml of boiling sulphuric acid. Very small pieces of sodium were added one at a time until the reaction mixture developed a blue color that persisted for 10 minutes. Ammonium chloride, 90 mg, was added. Then the ammonia was removed under reduced pressure from the frozen state. The residue was dissolved in 170 ml of water and the pH of the resulting solution was adjusted to approximately 6.5 by the addition of dilute ammonium hydroxide and dilute acetic acid. The solution at pH 6.5 was extracted five times with 50-ml portions of ether, the ether extracts being discarded. Then 10.0 ml of 0.011 N potassium ferricyanide were added dropwise to the aqueous solution with stirring. The pH was readjusted to approximately 6.5 by the addition of a few drops of dilute ammonium hydroxide, and the solution was allowed to stand at room temperature for 15 minutes. In order to remove the ferricyanide and ferrocyanide anions, the solution was passed through a column of AG 3-X4 anion exchange resin6 (100 to 200 mesh, 0.9 × 15 cm) in the chloride form. After the solution had passed through, the column was washed with 20 ml of water. The combined eluates were acidified by the addition of 1 ml of glacial acetic acid. In order to remove the remaining salts, the acidified solution was passed through a column (0.9 × 39 cm) of Amberlite IRC-50 resin in the hydrogen form (30). The resin column, to which the peptide product was bound at this point, was washed with 100 ml of 0.25% acetic acid. The peptide was eluted with a 30% pyridine-4% acetic acid solution. The eluate was collected in approximately 5-ml fractions and aliquots of each fraction were analyzed for peptide content by the Folin-Lowry color reaction (31). The peptide-containing fractions were combined and lyophilized. The residue was triturated in a mortar with six 5-ml portions of water. The water extracts were filtered, combined, and lyophilized.

The dry residue after lyophilization of the water extracts was dissolved in 2 ml of an ammonium acetate buffer that had been prepared by adding sufficient glacial acetic acid to 0.5 M ammonium acetate to lower the pH to 5.85. A 1-ml portion of the resulting solution was placed on a column (0.9 × 38 cm) of Amberlite IRC-50 that had been equilibrated previously with the buffer. Elution was performed with the same buffer at a flow rate of about 2.9 ml per hour, the effluent being collected in 1.9-ml fractions. Aliquots of each effluent fraction were analyzed by high performance liquid chromatography for the presence of the main peak. Most of the peptide emerged from the column as a single peak containing Fractions 10 through 18. A smaller amount of peptide was eluted earlier in a peak that overlapped slightly the main peak. Fractions 8 through 24 were combined and lyophilized. The dry residue was lyophilized twice more, the solid being dissolved each time in 15 ml of water. The second 1-ml portion of desalted peptide solution was subjected to the same purification and isolation procedure.

The combined products from the two identical purifications were dissolved again in 1 ml of the same buffer, placed on a single IRC-50 column, and subjected to a second chromatographic purification under the same conditions. The small peak that preceded the main peak was detected again although the size of the peak was much smaller than in the first purification. Fractions 10 through 24 were collected, Fractions 8 and 9 being discarded in order to minimize contamination of the material in the main peak by that in the smaller. The combined fractions were lyophilized three times. A white powder was obtained; weight, 32 mg; [α]$	ext{D}^{22}$ - 87.9° (c, 0.50 in N acetic acid). Portions of the product were subjected to strips of Whatman No. 1 paper and subjected to descending chromatography in butanol-acetic acid-water (4:1:5). The chromatograms were developed with a modified ninhydrin reagent (32), and also with a platinum reagent for the detection of sulfur-containing compounds (33). The peptide traveled as a single spot ($R_\text{f}$ 0.44 to 0.46). The $R_\text{f}$ of an authentic sample of 8-lysine-vasopressin under the same conditions was 0.23 to 0.28. Paper chromatographic analysis also was carried out in pyridine-acetic acid-water (50:35:15), the chromatograms being developed with the modified ninhydrin reagent. The peptide traveled as a single spot ($R_\text{f}$ 0.73). The $R_\text{f}$ of 8-lysine-vasopressin in this solvent system was 0.64.

A portion of the product was applied to a strip of Whatman No. 1 paper and subjected to electrophoresis at 2° for 16 hours at 400 volts in pyridine acetate buffer at pH 4.9. The paper was sprayed with the modified ninhydrin reagent. The 1-acetyl-8-lysine-vasopressin traveled a distance of 10.3 cm as a single spot toward the cathode. Under exactly the same conditions, a sample of 8-lysine-vasopressin traveled a distance of 16.7 cm in the same direction.

For elementary analysis, a sample of the product was heated for 5 hours at 100° in a high vacuum over phosphorus pentoxide. A loss in weight of 7% was observed. The results of the analysis agreed with the calculated values for the acetate of 1-acetyl-8-lysine-vasopressin.

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\text{C}_{19} \text{H}_{29} \text{O}_{8} \text{N}_{11} \text{S}_{2}
\]

**Calculated:** C 57.8, H 6.11, N 12.7

**Found:** C 57.5, H 6.13, N 12.6

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\text{C}_{19} \text{H}_{29} \text{O}_{8} \text{N}_{11} \text{S}_{2}
\]

**Calculated:** C 51.8, H 6.18, N 15.7

**Found:** C 51.5, H 6.38, N 15.8

A portion of the product was hydrolyzed in 6 N hydrochloric acid at 110° for 17 hours, and the amino acid content of the hydrolysate was determined (34, 35). All of the amino acids were present in the expected molar ratios. However, the analysis indicated the presence of an additional mole of ammonia and suggested that the product contained ammonium acetate which had not been removed completely by lyophilization.

In order to separate the 1-acetyl-8-lysine-vasopressin from this presumed contaminant and to obtain more information on the state of purity of the peptide, a portion of the product was subjected to countercurrent distribution. Chromatographically purified 1-acetyl-8-lysine-vasopressin, 60 mg, was dissolved in 6 ml of lower phase of the solvent system butanol ethanol 0.65% acetic acid (4:1:5). The solution was placed in the first two tubes of a 400-tube countercurrent distribution apparatus and subjected to 576 transfers in this solvent system. Analysis of aliquots by the Folin-Lowry color reaction indicated that essentially all of the peptide was present in tubes 101 to 165 as a single symmetrical peak ($K = 0.29$). A small amount of peptide which represented less than 3% of the amount in the main peak was present in tubes 80 to 100. The contents of tubes 101 to 165 were removed, combined, and concentrated in a rotary evaporator to a volume of about 20 ml. The concentrated

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6 Analytical grade Dowex 3 anion exchange resin supplied by Bio-Rad Laboratories, Richmond, California.
solution was lyophilized to a white, flinty powder; weight, 57 mg; [α]D = 94.8° (c, 0.50 in N acetic acid).

A portion of the material purified by countercurrent distribution was hydrolyzed and subjected to amino acid analysis under the same conditions used for the chromatographically purified product. The following molar ratios of amino acids and ammonia were obtained (with the value for phenylalanine taken as 1.0): phenylalanine, 1.0; lysine, 0.8; aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; tyrosine, 1.0; and ammonia, 2.9.

A sample of the product purified by countercurrent distribution was dried for elementary analysis under the same conditions used for the chromatographically purified material. A loss in weight of 8% was observed. The results of the analysis agreed with the calculated values for the acetate of 1-acetyl-S-lysine-vasopressin. Found: C 51.8, H 6.27, N 16.0.

The chromatographically purified product was used in all of the biological studies. This material may have contained about 6% of ammonium acetate and about 3% of peptide impurity as indicated by the high ammonia content, the separation of a small amount of peptide contaminant by countercurrent distribution, and the small increase in rotation after countercurrent distribution.

**SUMMARY**

A derivative of S-lysine-vasopressin in which the NH₂-terminal amino group contains an acetyl substituent has been synthesized. The protected nonapeptide N-acetyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-Asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-lysylglycinamide was prepared from the appropriate octapeptide derivative and p-nitrophenyl N-acetyl-S-benzyl-L-cysteinate. 1-Acetyl-S-lysine-vasopressin was prepared from the protected nonapeptide by first removing the benzyl groups from the lysine residue with sodium in liquid ammonia and then converting the resulting sulfhydryl peptide into the cyclic di-sulfide by potassium ferricyanide oxidation. The crude product was purified by ion exchange chromatography and countercurrent distribution.

Introduction of the acetyl group into the S-lysine-vasopressin molecule markedly reduced the biological activity of the hormone. The rat pressor and avian depressor effects were eliminated completely. The rat antidiuretic, rat oxytocic, and rabbit milk-ejecting activities were reduced to levels that were less than 0.5% of the corresponding activities of S-lysine-vasopressin. The acetylated hormone inhibited the rat pressor and avian depressor effects of the U.S.P. Posterior Pituitary Reference Standard, but did not inhibit the antidiuretic, oxytocic, or milk-ejecting effects of the standard.

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