England), unpublished observation.
(10) M. W. Miller, U.S. Patent 3 012 723; Oct 14, 1975.
(11) J. E. Lynch, *Am. J. Vet. Res.*, **22**, 324 (1961).
(12) L. R. Chappel, H. L. Howes, and J. E. Lynch, *J. Parasitol.*, **60**, 415 (1974).
(13) J. J. Rash and M. J. Lynch, *Drug Metab. Dispos.*, 4, 59 (1976).
(14) M. W. Miller, H. L. Howes, Jr., and A. R. English, *Antimicrob. Agents Chemother. (1961–70)*, 257 (1970).
(15) J. Ryley, R. Wilson, and M. Betts, *Parasitology*, **68**, 69 (1974).
(16) A. Kluge, J. Caroon, S. Unger, and J. Ryley, *J. Med. Chem.*, **21**, 529 (1978).
(17) M. Lynch and S. Figdor, *J. Agr. Food Chem.*, **25**, 1344 (1977).

[3-(1,4-Cyclohexadienyl)-L-alanine,8-lysine]vasopressin: Synthesis and Some Pharmacological Properties

Some Nath Banerjee, Lillian Diamond, Charlotte Ressler,*

Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06032

and Wilbur H. Sawyer

Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York 10032.
Received January 15, 1979

[3-(1,4-Cyclohexadienyl)-L-alanine,8-lysine]vasopressin, otherwise known as [3-(2,5-dihydrophenylalanine),8-lysine]vasopressin or [DiHPhe^3]lysine-vasopressin, has been synthesized in an attempt to utilize 2,5-dihydrophenylalanine (DiHPhe) to evaluate the contribution of aromaticity in position 3 to biological activity. The analogue has the same primary structure as lysine-vasopressin, except that two additional hydrogen atoms are present on the ring moiety of the phenylalanine residue in position 3. The key intermediate was the protected nonapeptide *N*-carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosyldihydrophenyl-L-alanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-*N*$^{\epsilon}$-tosyl-L-lysylglycinamide that was synthesized stepwise by the solid-phase technique. Deprotection with sodium in liquid ammonia was followed by sulfhydryl oxidation with I_2 to give the hormone analogue. [DiHPhe^3]lysine-vasopressin exhibited 125–130 units/mg of antidiuretic, 129–132 units/mg of rat pressor, and 6 units/mg of rat uterus contracting activity. To confirm the presence of DiHPhe in the analogue, an enzymatic procedure employing *Aspergillus oryzae* was developed that liberates in high yield the amino acid residue in position 3 of the posterior pituitary hormone structure. This study should be applicable to other biologically active peptides.

Interest in examining the biological peoperties of 2,5-dihydrophenylalanine [3-(1,4-cyclohexadienyl)-L-alanine, DiHPhe] in peptide form as an analogue of phenylalanine followed from the finding that DiHPhe is a highly effective antagonist of phenylalanine for the rat and a variety of bacteria.[1,2] Although the molecular basis of the antagonism of DiHPhe had not been explored at that time, the question arose whether substitution of this amino acid in a phenylalanine-containing peptide hormone would produce inhibitory properties that could be pharmacologically useful. Lysine-vasopressin (LVP), the antidiuretic hormone of the pig that has the structure Cys-Tyr-Phe-Gln-Asn--Asn-Cys-Pro-Lys-Gly-NH_2, seemed a good candidate for such a study. Substitution of DiHPhe for Phe in LVP had additional interest inasmuch as the substitution would be in residue-3, a position thought to be important in binding of neurohypophyseal hormones to their receptors.[3] In the three-dimensional structure proposed for neurohypophyseal hormones, the amino acid residue in position 3 is thought to occupy one of the corners of a β turn in a way that would allow its side chain to participate in intermolecular interactions.[4-6] Moreover, it is one of the positions (3, 4, and 8) undergoing substitution in the nine recognized naturally occurring oxytocic and antidiuretic neurohypophyseal peptides,[7] which suggested that this position confers some specificity to the biological properties.

In [DiHPhe^3]LVP the aromatic character of the ring of the phenylalanine residue that might contribute to the binding of LVP would be replaced by the π electron atmosphere of two isolated double bonds. As judged from values calculated from heats of hydrogenation for related olefins, the resonance energy of 1,4-cyclohexadiene is very low and similar to that of 1,3-cyclohexadiene: benzene, 36.0; 1,3-cyclohexadiene, 1.8; 1,3-cyclopentadiene, 2.9; 1,3-pentadiene, 4.2; 1,4-pentadiene, −0.2 kcal/mol. Heats of hydrogenation of 1,4-cyclohexadiene and 1,3-cyclohexadiene are −53.6 and −53.9 kcal/mol, respectively.[8,9] It can be accepted that 1,4-cyclohexadiene differs markedly from benzene in having little resonance energy and little diamagnetic ring current due to electron density. This is reflected, for example, in their relative effects in inducing shifts in the ^{1}H NMR spectrum of the dipole acetonitrile, which are 0.01 and 1 ppm, respectively.[10] This marked difference in ring current is expected to apply also to DiHPhe and Phe. In this respect, DiHPhe should have an advantage over β-2-thienylalanine which has recently served a similar purpose, i.e., to assess the contribution of aromaticity to biological activity by substitution for Phe in LVP.[11] The thiophene ring has about 70% of the resonance energy of the benzene ring.

Like Phe, DiHPhe is considered to have a planar ring and, differing only by 2 H atoms, it should have very similar molecular dimensions. In any comparison of peptides of DiHPhe and Phe to evaluate consequences of electronic differences, steric differences that might arise when other amino acids are substituted in this position, therefore, should be unimportant and need little consideration. In this respect, DiHPhe should have an advantage over β-cyclohexylalanine [Phe($3H_2$)], the cyclohexyl ring of which is puckered and nonplanar.[12] Past experiments designed to assess the contribution of aromaticity of Phe to biological activity by substitution of the Phe($3H_2$) residue include the synthesis of an analogue of the gastrin C-terminal tetrapeptide that had full biological activity[13,14] and [Phe($3H_2$)^3]oxytocin.[15] As compared to [Phe^3]-oxytocin, the analogue had a considerably lower potency in all oxytocin- and vasopressin-like activities observed but showed a greater intrinsic activity for the uterine receptor.

0022-2623/79/1822-1487$01.00/0

Substitution of Phe by DiHPhe in a peptide should be particularly appropriate when the corresponding substitution by another probe for aromaticity has resulted in a greatly diminished biological activity.

The relevance to activity of electron density at residue 7, another position proposed as a binding site in the posterior pituitary hormones, has recently been explored in a converse manner by substitution of the proline-7 residue by the 3,4-dehydroproline residue. In arginine-vasopressin, deaminoarginine-vasopressin, and oxytocin, this resulted in an approximate twofold increase in antidiuretic (ADH) activity.[16,17] This finding led to the synthesis of the very potent ADH analogue [1-deamino, 2-phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin.[18]

This article describes the synthesis of some pharmacological properties of [DiHPhe^3]LVP (Cys-Tyr-DiHPhe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2). Biological activities are considered in comparison with LVP and [8-lysine]vasotocin ([Ile^3]LVP, LVT). To facilitate analysis of DiHPhe in the hormone analogue, an enzymatic hydrolysis procedure was developed that is also presented.

Chemistry. As in similar early routes to oxytocin and vasopressin,[19,20] the key intermediate in the synthesis of [DiHPhe^3]LVP was the protected nonapeptide amide Z-Cys(Bzl)-Tyr(Bzl)-DiHPhe-Gln-Asn-Cys(Bzl)-Pro-Lys(ϵ-Tos)-Gly-NH_2 (II). This was synthesized stepwise by the Merrifield solid-phase method[21] as described by Stewart and Young.[22] It incorporated the modifications of Takashima and co-workers[23] and Manning[24] in the following manner. Boc-L-glutamine and the residues introduced after it were deprotected with trifluoroacetic acid (TFA). Boc-L-Pro, Boc-*S*-Bzl-L-Cys, Boc-*O*-Bzl-L-Tyr, and Cbz-*S*-Bzl-L-Cys were introduced by coupling with DCCI in methylene chloride. N^{α}-Boc-N^{ϵ}-Tos-L-Lys, Boc-L-Asn, Boc-L-Gln, and Boc-L-DiHPhe were introduced as their nitrophenyl esters by coupling in DMF. Ammonolysis of the resin-bound protected nonapeptide (I) liberated the desired protected nonapeptide as the amide (II).[25] This was precipitated from DMF as a solid by the addition of water. Protecting groups were then removed with sodium in liquid ammonia.[19,26] For conversion of the sulfhydryl peptide to the cyclic disulfide, a mild and rapid oxidative procedure was sought. Aeration in dilute aqueous solution that requires prolonged periods, as used in early syntheses of posterior pituitary hormones and analogues, was avoided because of the tendency of DiHPhe to undergo oxidation and autoxidation to phenylalanine.[1,27] Potassium ferricyanide, oxidant in the synthesis of many later analogues, led to a product that retained a yellow color in preliminary work and was not further pursued. Oxidation with I_2 in dilute acetic acid was next undertaken. A heptapeptide precursor of arginine-vasopressin had been cyclized oxidatively at the cysteine moieties with I_2 in 80% acetic acid.[28] However, having two isolated double bonds, DiHPhe could well be susceptible to iodination. After being examined for possible side reactions, the procedure was considered suitable for accomplishing disulfide ring closure of deprotected II. (That DiHPhe and Tyr were not iodinated to a significant extent was indicated by the high recoveries of these two amino acids on enzymatic hydrolysis of the nonapeptide. Yields ranged from 75 to 90% and 91 to 96%, respectively. That little over-oxidation of the cystinyl to the cysteyl residue took place with the I_2 reagent was confirmed by the quantitative consumption of I_2 and the expected molar cystine content of the nonapeptide formed by such treatment. Moreover, when equimolar mixtures of cysteine and dihydrophenylalanine were treated with I_2, 100% cystine formed and 88% dihydrophenylalanine was recovered unchanged.)

The crude [DiHPhe^3]LVP product formed by such oxidative treatment was freed of salts on a column of Amberlite IRC-50. It was purified by chromatography[29] on the same ion-exchange resin in the manner described for synthetic LVP.[30] When chromatographed on the analytical resin column,[29] [DiHPhe^3]LVP eluted as a single substance in the same position as a sample of synthetic LVP. As might be expected, its behavior on TLC and paper electrophoresis was also very similar to that of LVP. In separated well from LVP on reverse-phase chromatography.

Enzymatic Hydrolysis of [DiHPhe^3]LVP, Neurohypophyseal Hormones, and Related Compounds. Because of the lability of DiHPhe under usual acidic and alkaline conditions of hydrolysis,[1] analysis of the composition of [DiHPhe^3]LVP and the corresponding protected nonapeptide posed a special problem. Although frequently very useful for determining DiHPhe in simple derivatives and peptides, quantitative analysis of Phe-DiHPhe content by NMR[1] is less suitable for larger peptides. In general, the posterior pituitary hormones have been considered to be notably resistant to quantitative enzymatic digestion, and no method has been available for this purpose. For example, leucinaminopeptidase is only slightly active toward oxytocin.[31] Papain was used by Lawler, du Vigneaud, and colleagues[32] to obtain direct evidence for asparagine and glutamine in natural oxytocin, but yields were low and most products were unspecified. Limited enzymatic cleavage of the posterior pituitary hormones results at specific sites with chymotrypsin, trypsin, hemicystinyltyrosine peptidase, and preparations from various organ tissues. A recently recognized mammalian kidney endopeptidase cleaves oxytocin and arginine-vasopressin at the proline carboxyl linkage.[33] (For a review of the action of various enzymes on oxytocin and vasopressin, see ref 34.)

With substrates such as synthetic ribonuclease S fragments, aminopeptidase M was used profitably by Hofmann et al. to establish stereochemical purity as judged by the completeness of hydrolysis.[35] Pronase, followed by aminopeptidase M, was employed by Bennett et al.[36] for structural studies on human γG immunoglobulin, particularly for locating asparagine and glutamine residues.

In the present study, attempts were made to develop an enzymatic procedure to liberate effectively from the newly synthesized DiHPhe analogue the DiHPhe residue in position 3. Pronase and aminopeptidase M were employed. In addition, *Aspergillus oryzae* was examined since it is more active than kidney acylase in deacetylating derivatives of aromatic amino acids.[37] Papain, followed by aminopeptidase M, was also investigated. Used as models for the enzymatic degradation were LVP, oxytocin, and performic acid oxidized oxytocin ($CySO_3H$-Tyr-Ile-Gln-Asn-$CySO_3H$-Pro-Leu-Gly-NH_2). Like the latter, the disulfhydryl form was expected to be more accessible than the native hormones to enzymatic attack, and the intact hormones were therefore treated first with an excess of 2-mercaptoethanol to cleave the disulfide bond. Pronase was quite effective in degrading oxytocin, and when followed by aminopeptidase M it was useful for performic acid oxidized oxytocin. In both instances, the average yield of most constituent amino acids was 55%. Under the conditions tried, the action of papain, followed by aminopeptidase M, was slow and was not further studied. *Aspergillus oryzae* liberated Ile from position 3 of oxytocin and performic acid oxidized oxytocin in 75 and 72% yield, respectively. *Aspergillus oryzae* was particularly effective

Table I. Some Pharmacological Activities of [3-(1,4-Cyclohexadienyl)-L-alanine,8-lysine]vasopressin and Relevant Hormones and Analogues

	activity, units/mg			
	uterus (rat)	vasodepressor (chicken)	ADH (rat)	vasopressor (rat)
	lysine-vasopressin			
[Phe^3,Lys^8]VP[a]	9.5 ± 0.4		212 ± 13	258 ± 5
[Phe^3,Lys^8]VP[b]	5	40	246	270
	lysine-vasotocin			
[Ile^3]LVP[c]		190		130
[Ile^3]LVP[d]	78	210	24	130
[Thi^3]LVP[e]	19.0 ± 0.5	87 ± 4	332 ± 32	243 ± 5
[Ser^3]LVP[b]	<0.01	<0.01	~0.08	<0.01
[Tyr^3]LVP[b]	0.01	0.1	0.18	1.6
[Tyr^3]LVP[b]	<0.01	0.08		0.07
[DiHPhe^3]LVP[f]	6.3 ± 0.4		137 ± 11	137 ± 9
[DiHPhe^3]LVP[g,h]	6		125-130	129-132
	arginine-vasopressin			
[Phe^3,Arg^8]VP[b]	16	57	429	380
	arginine-vasotocin			
[Ile^3,Arg^8]VP[b]	114	286	245	243

[a] Material furnished by J. Lowbridge and M. Manning. [b] Data tabulated in ref 38 corrected to units/mg. [c] Reference 39. [d] Reference 40. [e] Reference 11. [f] Observed uncorrected values. [g] Corrected for 6-9% LVP. [h] Structures of [DiHPhe^3]-LVP and LVP are given under the Introduction.

for vasopressin. Phenylalanine-3, tyrosine-2, and lysine-8 were liberated quantitatively, and the overall yield of most other amino acids was also high.

Degradation with *Aspergillus oryzae* was carried out at 37 °C for 22 h. With DiHPhe-containing peptides, the digestion mixture included 1,4-cyclohexadiene, which succeeded in retarding oxidation of DiHPhe to Phe during the degradation although it decreased the overall yield somewhat. Correction was made for Phe formed from DiHPhe and liberated from the enzyme by means of a separate digest, consisting of constituent amino acids and enzyme. The protected [DiHPhe^3,Lys^8]-nonapeptide II was deprotected with Na/NH_3. After removal of the NH_3, the formed disulfhydryl nonapeptide in aqueous solution was treated with the *Aspergillus* preparation. Purified [DiHPhe^3]LVP was treated directly with this enzyme preparation and, surprisingly, it did not require prior reductive treatment to the disulfhydryl compound.

Starting Boc-L-DiHPhe had 3% Phe, as determined by ^{1}H NMR. On enzymatic hydrolysis, newly synthesized protected nonapeptide II showed 6% Phe in residue 3. After storage of II for 5 years over silica gel at 5 °C, 7.8% Phe was present in this residue. It is clear that DiHPhe can be incorporated into large peptides by the solid-phase technique without serious oxidation and, apparently, it is reasonably stable to prolonged storage in a protected form. Nevertheless, stability of DiHPhe should be verified when DiHPhe is incorporated into other peptides. [DiHPhe^3]-LVP derived from II and purified by anion-exchange chromatography had 11.6% Phe in residue 3. Such estimates of Phe are approximate, since each Phe value is the remainder from two amino acid analyses. They are also considered to be maximal, since DiHPhe/Phe ratios tended to increase with the degree of hydrolysis, which was 57 to 75%. Morever, direct acid hydrolysis of [DiHPhe^3]LVP showed only 6% Phe. For purposes of calculating the biological activities of the analogue, LVP contamination was estimated to be 6–9%.

Biological Results and Discussion. Potencies of [DiHPhe^3]LVP and relevant analogues are given in Table I. [DiHPhe^3]LVP showed considerable pharmacological activity in vivo in the rat. Antidiuretic activity was 125–130 units/mg and pressor activity was 129–132 units/mg. Activity on the isolated rat uterine strip was 6 units/mg. These values are 4–7% lower than the observed activities which were corrected for 6–9% LVP, respectively, estimated to be present on the basis of Phe content. [DiHPhe^3]LVP thus appears to behave as an effective agonist with properties similar to those of LVP. That an amino acid with antimetabolite properties in vivo can serve to form a peptide hormone analogue with agonist properties is acceptable, especially in view of the consideration that separate processes with distinct mechanisms of action are likely for the antimetabolite and hormone effects. Moreover, since this work was undertaken, ample precedent has accumulated, for example, in the synthesis of β-2-thienylalanine-containing analogues of bradykinin[41] and LVP[11] that are biologically active.

In general, analogue substitution is likely to result in a change in the ratio of the several pharmacological activities residing within a single molecule. Therefore, it was of considerable interest to note that the ratio in [DiHPhe^3]-LVP of the three determined activities remained close to that of LVP. The close similarity in the molecular dimensions of Phe and DiHPhe may be important in this regard. However, the data are consistent also with the suggestion that [DiHPhe^3]LVP is converted in vivo to LVP. Such a possibility gains no support from the consideration that it is unlikely that the same degree of conversion of [DiHPhe^3]LVP to LVP would take place in an isolated tissue preparation, as after intravenous injection. Moreover, some evidence for the biological stability of DiHPhe is provided by the observation that DiHPhe can be incorporated extensively into protein of certain *Escherichia coli* and sarcoma cells.[42] In addition, when DiHPhe was treated in phosphate buffer, pH 7.5, with homogenates of rat kidney and liver, no significant conversion to Phe was detected. Three other synthetic analogues of LVP bearing substitutions in position 3 (serine, tyrosine, or tryptophan) have extremely low pharmacological activities. However, lysine-vasotocin, having a 3-isoleucine substitution, possesses appreciable vasodepressor activity, and [3-β-thienyl-L-alanine,8-lysine]vasopressin possesses appreciable rat pressor and antidiuretic activities. Thus, the findings with [DiHPhe^3]LVP could well represent intrinsic pharmacological activity for this analogue,

reflecting binding of the analogue to the same receptors as those that bind LVP. Consistent with this concept are the findings of a recent separate study in which L-DiHPhe was found to substitute for Phe as a substrate for L-phenylalanine ammonia-lyase, thereby giving rise to 3-(2,5-dihydrophenyl)acrylate in place of cinnamate.[43] This type of elimination reaction generally requires activation by an electron-withdrawing substituent in the β position, which can be a carbonyl, ester, thioester, or aromatic moiety. Apparently, the π electrons of the γ–δ double bond provide sufficient β activation to replace the aromatic system of Phe. The Michaelis constant for L-DiHPhe as compared to L-Phe in the enzymatic elimination of ammonia was lowered only slightly, thus reflecting a similar degree of binding of the two substrates. The catalytic constant, k_{cat}, for this reaction of DiHPhe was reduced at least 14-fold as compared to Phe. This was attributed to an induced fit of the analogue to the enzyme with some resultant misalignment with respect to catalytic groups.

Oxytocic activity of [DiHPhe^3]LVP was low, about 64% that of LVP and only one-twelfth that of LVT. Thus, while compatible with oxytocic activity, electron density in position 3 does not appear to favor it. However, spatial requirements for oxytocic activity at position 3 need consideration in this instance. The rat vasopressor activity of [DiHPhe^3]LVP is about half that of LVP and about the same as LVT. Clearly, aromaticity or other electron density is not important for both the expression of this biological activity in LVP and for effective activation, although aromaticity appears to enhance activity. It is noted also that the spatial requirements at position 3 are not very restrictive for the vasopressor receptor that apparently is equally responsive to the somewhat dissimilar [Ile^3]- and [DiHPhe^3]LVP. By contrast, antidiuretic activity is about 62% that of LVP but seven times that of LVT. From the earlier studies with LVT and arginine-vasoctocin [Ile^3,Arg^8]VP (AVT), it is clear that electron density in position 3 and aromaticity are not essential for expression of ADH activity. π-Electron density in that position in LVP probably serves to enhance the activity. Clearly, aromaticity is not required for very effective ADH activity. This study modifies the current model of the biologically active conformation of lysine-vasopressin[44] by eliminating aromaticity in residue 3 as a requisite for very effective antidiuretic potency. Probably some π-electron density at this locus is sufficient.

The availability of [DiHPhe^3]LVP should make it possible to evaluate the importance of π–π interactions between phenylalanine-3 and the adjacent tyrosine-2 residue which is considered to be relevant in enhancing antidiuretic activity in this model. This work should also point to a similar approach to studying other biologically active peptides having phenylalanine in a critical position for binding with the receptor or when involved in conformationally significant interactions.

The enzymatic hydrolysis procedure developed provides a convenient means of degrading the characteristic posterior pituitary hormone cyclic disulfide structure. It should facilitate the synthesis and analysis of 2,5-dihydrophenylalanine-containing peptides.

Experimental Section

Chloromethylated copolystyrene–2% divinylbenzene resin containing 2.0 mequiv of Cl^-/g of resin, the reaction vessel, and mechanical shaker were obtained from Schwarz/Mann Biochemicals, Orangeburg, N.Y. Glacial acetic acid (AcOH), triethylamine (NEt_3), and methanol were redistilled and dimethylformamide (DMF) was distilled in vacuo and analyzed prior to use.[22] Boc-Gly, Boc-L-Pro, *S*-Bzl-L-Cys, Boc-L-Asn, Boc-L-Gln *p*-nitrophenyl ester, Boc-*O*-Bzl-L-Tyr, and Cbz-*S*-Bzl-L-Cys were purchased from Fluka, Switzerland. Boc-*S*-Bzl-L-Cys,[45] Boc-L-Asn *p*-nitrophenyl ester,[46] N^{α}-Boc-N^{ϵ}-Tos-L-Lys *p*-nitrophenyl ester,[47] and Boc-L-DiHPhe *p*-nitrophenyl ester[48] were synthesized by described procedures. Purity of these derivatives was checked by melting point and thin-layer chromatography.[22] Amino acid analyses were performed on a Beckman-Spinco automatic amino acid analyzer, Model 120.[49] Optical rotation was taken in a 2-dm cell in a Rudolph photoelectric spectropolarimeter system, Model 80ϕ 6-34402. Synthetic oxytocin and performic acid oxidized oxytocin prepared from natural oxytocin were available from previous studies.[19,50] Highly purified lyophilized samples of synthetic LVP were gifts of Drs. Johannes Meienhofer and Maurice Manning. A solution of LVP, 100 IU/mg, was purchased from Sigma Chemical Co., St. Louis, Mo. Aminopeptidase M was from Rohm and Haas, Darmstadt; Pronase was from Sigma. *Aspergillus oryzae* was a crude powder offered as α-amylase by Sigma. Papain was a gift of Dr. Gary Bailin. It had been activated previously by exhaustive dialysis against 5 mM cysteine in 2 mM EDTA.

Peptide Synthesis. Z-Cys(Bz)-Tyr(Bzl)-DiHPhe-Gln-Asn-Cys(Bzl)-Pro-Lys(ϵ-Tos)-Gly-Resin (I). Boc-Gly (1.0 g) was esterified to 10 g of resin following standard procedures,[22] giving material substituted with 0.2 mmol of Gly/g of resin. Each amino acid was added to the growing chain according to the following sequence of washes and reactions with use of 8 mL of solvent/g of resin: (1 and 2) three washes with ethanol, followed by three washes with AcOH; (3) (a) 1.2 N HCl in AcOH for 30 min or (b) TFA for 20 min for Gln, DiHPhe, and amino acids following DiHPhe; (4–6) three washes with AcOH, followed by three washes with EtOH, followed by three washes with $CHCl_3$; (7) 10% NEt_3–$CHCl_3$ for 10 min; (8) three washes with $CHCl_3$; (9) (a) three washes with CH_2Cl_2 (for DCCI coupling) or (b) three washes with DMF (for active esters); (10) (a) addition of 3 equiv of Boc-protected amino acid in a minimal volume of CH_2Cl_2 for 10 min, followed by 3 equiv of DCCI in CH_2Cl_2 (50%, w/v; reaction for 4 h), or (b) 3 equiv of Boc-protected amino acid NPE in a minimal volume of DMF (reaction for 18 h); (11) three washes with (a) CH_2Cl_2 or (b) DMF. After the last amino acid had been added, steps 1–8 were repeated. The peptide resin was removed from the vessel with the aid of EtOH and dried in vacuo over P_2O_5: yield 7.8 g from 6 g of Boc-Gly resin.

Z-Cys(Bzl)-Tyr(Bzl)-DiHPhe-Gln-Asn-Cys(Bzl)-Pro-Lys(ϵ-Tos)-Gly-NH_2 (II). A suspension of the resin nonapeptide (3.9 g) in 40 mL of MeOH stirred in an ice–salt bath was saturated with NH_3 that had been redistilled over sodium. The mixture was stirred for 18 h at 4 °C and then for 1 h at room temperature. The mixture was concentrated, fresh MeOH was added, and this was then removed. The latter procedure was repeated twice more. The final resin concentrate was stirred in 60 mL of DMF for 1 h and filtered, and the resin was washed well with three 20-mL portions of DMF, followed by three 20-mL portions of MeOH. The combined filtrate and washings were concentrated to a minimal volume (4 mL) to which 10 mL of water was then added. The white solid that precipitated was filtered off, washed with water, dried, triturated with boiling MeOH, and dried in vacuo over P_2O_5: yield 1.03 g as the combined product of two runs (53% based on Boc-Gly esterified to the resin).

[3-(1,4-Cyclohexadienyl)-L-alanine,8-lysine]vasopressin. A solution of II (100 mg, 61.8 μmol) in 8 mL of liquid NH_3 (freshly distilled over Na) was treated with a stick of Na encased in a narrow bore glass tube until the blue color persisted for at least 30 s.[19] Ammonium chloride (23 mg) was added, and NH_3 was removed at the water aspirator and finally under a current of dry N_2.

The white powder was dissolved in 80 mL of 1 N AcOH. The solution was cooled in a dry ice–acetone bath until it became a semisolid slurry. With vigorous stirring, a solution of I_2 (9.5 mM, 13.5 mL, 99.8%) in 0.25% KI was added dropwise as each drop was decolorized. At the earliest appearance of a faint yellow color throughout the solution, it was frozen and lyophilized.

The light yellow powder was dissolved in 2 mL of H_2O. The solution was adjusted to pH 4 and applied to a column (1 × 22 cm) of Amberlite CG-50 resin (200–400 mesh, Fischer Scientific Co.). The column was washed slowly with 0.25% AcOH. When flame tests for Na^+ and K^+ and a Nessler test for NH_4^+ in the effluent became negative, 30% pyridine–4% AcOH was introduced as eluent. The peptide was eluted at the solvent front, as indicated

Table II. Enzymatic Hydrolysis of [3-(1,4-Cyclohexadienyl)-L-alanine,8-lysine]vasopressin and Related Compounds

peptide	enzyme	amino acid content of hydrolysate,[d] mol %				
		Ile/Phe + DiHPhe	Tyr	Leu/ Lys	Gly	Asn + Gln
performic acid oxidized oxytocin	pronase	38		46	44	86
	+ aminopeptidase M	65		65	54	124
$CySO_3H$-Tyr-Ile-Gln-Asn-$CySO_3H$-Pro-Leu-Gly-NH_2[a]	α-amylase prepn	75	26	72	50	72
	+ aminopeptidase M	82	35	85	60	90
	+ cyclohexadiene	51	23	57	38	86
	papain + aminopeptidase M	13	30	14	13	14
oxytocin[a]	pronase	54	70	60	36	76
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2[b]	α-amylase prepn	72	63	77	63	110
lysine-vasopressin[a,c]	α-amylase prepn	113	100	101	73	102
Z-Cys(Bzl)-Tyr(Bzl)-DiHPhe-Gln-Asn-Cys(Bzl)-Pro-Lys(ε-Tos)-Gly-NH_2[b]	α-amylase prepn + cyclohexadiene	57.6 DiHPhe 3.9 Phe	77	nd	18	67
		52.4 DiHPhe 4.4 Phe	73	nd	nd	nd
[DiHPhe^3]lysine-vasopressin[c]	α-amylase prepn + cyclohexadiene	66 DiHPhe 8.7 Phe	89	nd	nd	nd

[a] Prior to treatment with enzyme, 10 μL of a 1% mercaptoethanol solution was added and the mixture was held at 5 °C for 1 h. [b] Peptide was pretreated with Na/NH_3. [c] Structures of [DiHPhe^3]LVP and LVP are given under the Introduction. nd = not determined.

by a positive Folin–Lowry reaction.[51] The desalted material was concentrated and lyophilized, yield 71 mg. It was then applied in 10 mL to a column (1.5 × 60.5 cm) of Amberlite CG-50 resin previously equilibrated with NH_4OAc (0.5 N, pH 6.38). The column was eluted with the same buffer at 25.5 °C. The flow rate was 12 mL/h, and 5-mL fractions were collected. Material within 170 and 248 mL was concentrated and desalted as before to furnish 17 mg of a white fluffy powder: $[\alpha]^{25}_D$ −30.2 ± 3° (*c* 0.12, 1 N AcOH), lit.[52] for LVP $[\alpha]^{21.5}_D$ −23.0° (*c* 0.5).

When chromatographed on a 0.9 × 24 cm analytical column of Amberlite IRC-50,[29] the material eluted in the same position as a sample of synthetic LVP. On electrophoresis on Whatman No. 1 paper in phosphate–NaCl buffer, pH 6, 0.1 μ, at 19 V/cm at 20 °C for 7 h, the material (0.3 μmol) moved as a single substance with a mobility (6.95 cm) close to that of LVP (7.0 cm), as detected with 0.1% ninhydrin in acetone. Paper electrophoresis of [DiHPhe^3]LVP (0.08 μmol) in pyridine–acetate buffer, pH 5.6, or potassium carbonate buffer, pH 9.3, for 12.6 h at 4 °C also showed in each system a single substance with a mobility of 2.7 and 11.9 cm, respectively, as detected with both the platinum[53] and bromphenol blue[54] reagents. TLC on silica gel H (Stahl) of [DiHPhe^3]LVP, LVP (0.1 μmol each), and a mixture of them (0.05 μmol each) was carried out by developing in the upper layer of 1-BuOH–AcOH–H_2O (4:1:5) and drying three consecutive times. Mobilities of the analogue and LVP were the same (R_f 0.31), and no separation of the mixture resulted as detected with the platinum reagent.[53] However, [DiHPhe^3]LVP separated well from LVP on a Waters 30 × 0.4 cm μBondapak C_{18} (10 μm) analytical column in the system 20% CH_3CN–0.01 M potassium phosphate buffer, pH 7.0. With a flow rate of 1 mL/min, elution volumes were 38.7 and 31.6 mL, respectively.

A very broad peak also eluted from the preparative Amberlite CG-50 resin column between 390 and 700 mL, which when treated similarly furnished 19 mg. Electrophoresis showed the presence of four materials in the region 6.2 to 8.2 cm from the origin which were not further purified.

Amino acid analysis of the product after hydrolysis for 20 h in 6 N HCl at 110 °C gave an 89% recovery in the following molar ratios: Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; $^1/_2$-Cys, 1.8; Tyr, 0.6; Lys, 1.0; NH_3, 3.1; Phe, 0.06. A sample subjected to performic acid oxidation[55] and then hydrolyzed in the same manner gave the following amino acid ratios: Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; Phe, 0.5; $CySO_3H$, 2.0; Lys and NH_3, not determined.

Enzymatic Hydrolyses. Pronase digestions were carried out in 0.2 M potassium phosphate buffer, pH 7.7, at a ratio of substrate to enzyme of 5:1. Half the enzyme was added initially and half after 18 h at 37 °C, and incubation was continued for 4 h. Aminopeptidase M digestions were carried out in 0.2 M potassium phosphate buffer, pH 7.7, at a ratio of substrate to enzyme of 1:1.5 for 3 h at 37 °C. Papain digestions were carried out in 0.5 M ammonium acetate buffer, pH 5.3, in the presence of 0.15% mercaptoethanol at a ratio of substrate to enzyme of 20:1 for 6 h at 37 °C. Digests were acidified, clarified, and lyophilized before being treated subsequently with aminopeptidase M.

***Aspergillus oryzae* Digestions. General Procedure.** To a solution of 0.9 μmol of peptide in 150 μL of potassium phosphate buffer, pH 7, was added 1 mg of α-amylase preparation in an equal part of buffer. 1,4-Cyclohexadiene (10 μL) and toluene (1 drop) were added, and the mixture was incubated under N_2 at 37 °C for 17 h. A control consisting of a mixture of the constituent amino acids, cyclohexadiene, enzyme, and buffer was treated in the same manner. The mixtures were then adjusted to pH 2.2 and subjected to amino acid analysis. Percent Phe formed in the control was subtracted from percent Phe in the liberated DiHPhe plus Phe, to give percent Phe in residue 3.

The protected peptide amide II (3.35 mg, 2.07 μmol) was treated with sodium in liquid NH_3 prior to being treated with 2 mg of α-amylase in the manner just described. After storage for 5 years at 5 °C over silica gel, II was treated with sodium and was reanalyzed. Prior to enzymatic digestion, oxytocin and LVP were pretreated with 2-mercaptoethanol, and PAOO and [DiHPhe^3]-LVP received no treatment. Amino acid analyses of the digests are given in Table II.

Bioassay Methods. Oxytocic activity was determined on the isolated rat uterus suspended in a Mg^{2+}-free van Dyke–Hastings solution,[56] antidiuretic activity by intravenous injection into water-loaded rats under ethanol anesthesia,[57] and vasopressor activity in phenoxybenzamine-treated rats under urethane anesthesia.[58] The USP Posterior Pituitary Reference Standard was used in all assays. A preliminary report of this work has been made.[59]

Acknowledgment. This work was supported by Grants NS 04316, NS 12448 (CR), AM 01940, and HL 12738 (W.H.S.). Part of this work was carried out at the Institute for Muscle Disease, New York, N.Y. The authors thank Drs. Johannes Meienhofer and Maurice Manning for gifts of lysine-vasopressin, Mrs. Jane Salzman for capable assistance with enzyme studies, and Dr. Walter Chan of Cornell University Medical College for preliminary pressor bioassays.

References and Notes

(1) M. L. Snow, C. Lauinger, and C. Ressler, *J. Org. Chem.*, **33**, 1774 (1968).
(2) D. S. Genghof, *Can. J. Microbiol.*, **16**, 545 (1970).
(3) J. Rudinger and Krejci, *Experientia*, **18**, 585 (1962).
(4) D. W. Urry and R. Walter, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 956 (1971).
(5) P. H. Von Dreele, A. I. Brewster, F. A. Bovey, H. A. Sheraga, M. F. Ferger, and V. du Vigneaud, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 3088 (1971).
(6) R. Walter, I. L. Schwartz, J. H. Darnell, and D. W. Urry, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1355 (1971).
(7) R. Acher, J. Chauvet, and M.-T. Chauvet, *Eur. J. Biochem.*, **40**, 585 (1973).
(8) R. D. Gilliom, in "Introduction to Physical Organic Chemistry", Addison-Wesley, Menlo Park, CA, 1970, p 70.
(9) R. B. Turner, B. J. Mallon, M. Tichy, W. von E. Doering, W. R. Roth, and G. Schröder, *J. Am. Chem. Soc.*, **95**, 8605 (1973).
(10) F. A. L. Anet and G. E. Schenck, *J. Am. Chem. Soc.*, **93**, 556 (1971).
(11) C. W. Smith, M. F. Ferger, and W. Y. Chan, *J. Med. Chem.*, **18**, 822 (1975).
(12) E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, in "Conformational Analysis", Interscience, New York, 1966, p 42.
(13) H. Gregory, D. S. Jones, and J. S. Morley, *J. Chem. Soc. C*, 531 (1968).
(14) J. S. Morley, *Proc. R. Soc. London, Ser. B*, **170**, 97 (1968).
(15) R. Walter, C. W. Smith, and J. Roy, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3054 (1976).
(16) C. W. Smith, C. R. Botos, and R. Walter, *Pept., Proc. Am. Pept. Symp., 5th, 1977*, 161 (1977).
(17) S. Moore, A. M. Felix, J. Meienhofer, C. W. Smith, and R. Walter, *J. Med. Chem.*, **20**, 495 (1977).
(18) C. W. Smith and R. Walter, *Science*, **199**, 297 (1978).
(19) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 3115 (1954).
(20) V. du Vigneaud, M. F. Bartlett, and A. J. Jöhl, *J. Am. Chem. Soc.*, **79**, 5572 (1957).
(21) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).
(22) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, 1969.
(23) H. Takashima, V. du Vigneaud, and R. B. Merrifield, *J. Am. Chem. Soc.*, **90**, 1323 (1968).
(24) M. Manning, *J. Am. Chem. Soc.*, **90**, 1348 (1968).
(25) M. Bodanszky and J. T. Sheehan, *Chem. Ind. (London)*, 1423 (1964).
(26) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
(27) C. Ressler, *J. Org. Chem.*, **37**, 2933 (1972).
(28) D. A. Jones, Jr., R. A. Mikulek, and R. H. Mazur, *J. Org. Chem.*, **38**, 2865 (1973).
(29) A. Light, R. Acher, and V. du Vigneaud, *J. Biol. Chem.*, **228**, 633 (1957).
(30) J. Meienhofer and Y. Sano, *J. Am. Chem. Soc.*, **90**, 2996 (1968).
(31) R. L. Hill and E. L. Smith, *J. Biol. Chem.*, **228**, 577 (1957).
(32) H. C. Lawler, S. P. Taylor, A. M. Swan, and V. du Vigneaud, *Proc. Soc. Exp. Biol. Med.*, **87**, 550 (1954).
(33) M. Koida and R. Walter, *J. Biol. Chem.*, **251**, 7593 (1976).
(34) C. Ressler and E. A. Popenoe, *Methods Invest. Diagn. Endocrinol.*, **2A**, 681–691 (1973).
(35) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).
(36) C. Bennett, W. H. Konigsberg, and G. M. Edelman, *Biochemistry*, **9**, 3181 (1970).
(37) I. Chibata; see J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids", Vol. II, Wiley, New York, 1961, pp 1771 and 1772.
(38) B. Berde and R. A. Boissonnas, *Handb. Exp. Pharmacol.*, **23**, 802 (1968).
(39) R. D. Kimbrough and V. du Vigneaud, *J. Biol. Chem.*, **236**, 778 (1961).
(40) R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, **45**, 1629 (1962).
(41) F. W. Dunn and J. M. Stewart, *J. Med. Chem.*, **14**, 779 (1971).
(42) M. J. Pine, *Antimicrob. Agents Chemother.*, 601 (1975).
(43) K. R. Hanson, E. A. Havir, and C. Ressler, *Biochemistry*, **18**, 1431 (1979).
(44) R. Walter, C. W. Smith, P. K. Mehta, S. Boonjarern, J. A. L. Arruda, and N. A. Kurtzman, in "Disturbances in Body Fluid Osmolality", T. E. Andreoli, J. J. Grantham, and F. C. Rector, Jr., American Physiological Society, Bethesda, Md., pp 1–37.
(45) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 186 (1967).
(46) E. Schröder and E. Kleiger, *Justus Liebigs Ann. Chem.*, **673**, 208 (1964).
(47) C. H. Li, J. Ramachandran, and B. Gorup, *J. Am. Chem. Soc.*, **86**, 2703 (1964).
(48) G. R. Nagarajan, L. D. Diamond, and C. Ressler, *J. Org. Chem.*, **38**, 621 (1973).
(49) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(50) C. Ressler and V. du Vigneaud, *J. Biol. Chem.*, **211**, 809 (1954).
(51) O. H. Lowry, J. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(52) J. Meienhofer and V. du Vigneaud, *J. Am. Chem. Soc.*, **82**, 2279 (1960).
(53) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).
(54) I. I. Geschwind and C. H. Li, *J. Am. Chem. Soc.*, **74**, 834 (1952).
(55) S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).
(56) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(57) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(58) J. Dekanski, *Br. J. Pharmacol.*, **7**, 567 (1952).
(59) C. Ressler, S. N. Banerjee, M. Tsutsumi, L. Diamond, and W. H. Sawyer, *Pept., Proc. Am. Pept. Symp., 6th, 1979*, in press.