Synthesis and Hormonal Activities of 8-L-Homonorleucine-vasopressin

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An analog of lysine-vasopressin in which the ε-amino group of the lysine residue is replaced by a methyl group has been prepared. In the rat it shows about 8% of the pressor effect and about 4% of the antidiuretic activity of lysine-vasopressin. The synthesis was carried out in solution by the stepwise approach. The usefulness of o-nitrophenyl esters in peptide synthesis was examined.

In studies of synthetic analogs of lysine-vasopressin, the lysine residue containing the only side-chain amino group in the natural compound has been the subject of continued interest.[1] In one analog, 8-formyllysine-vasopressin,[2] the basic amino group of the lysine side chain is masked by acylation. Such an introduction of an additional group could produce misleading results. For instance, the biological properties of 2-O-methyltyrosine-oxytocin[3] could suggest that the hydroxyl group is necessary for activity, yet 2-phenylalanine-oxytocin[4,5] has significant biological activity. Thus, the introduction of the methyl group, and not the removal of the hydroxyl, is responsible for the drastic loss in potency. The pharmacological properties of a vasopressin analog in which lysine was replaced by leucine were reported in the literature.[6,7] However, because of the branching in the side chain of leucine potentially misleading conclusions could be drawn concerning the role of the straight-chain lysine residue. For a conclusive examination of the role of the side-chain amino function in lysine vasopressin, its replacement by either hydrogen† or a methyl group should be considered. To keep the length of the side chain comparable to that of the lysine residue, we decided to synthesize 8-L-homonorleucine-vasopressin, an analog in which a methyl group replaces the amino group of lysine.

Homonorleucine (DL) was synthesized as described in the literature.[8] Initially, preparation of the desired analog from DL-homonorleucine and separation of the diastereoisomers at the tripeptide stage were attempted.[9] However, separation of Z-L-Pro-L-Hnl-Gly-OEt from Z-L-Pro-D-Hnl-Gly-OEt was achieved neither by crystallization nor by countercurrent distribution. A continuation of the synthesis through the protected nonapeptide yielded, after deprotection and cyclization, a complex mixture.‡ Because of these difficulties the DL-amino acid was resolved in the form of its acetyl derivative with hog renal acylase.[10] Benzyloxycarbonyl-L-homonorleucine was coupled to glycine ethyl ester by the trimethylacetic acid mixed anhydride method.[11,12] After deprotection, the dipeptide ester was acylated with the p-nitrophenyl ester of benzyloxycarbonyl-L-proline,[13] and the protected tripeptide ester was treated with methanolic ammonia to form the corresponding amide. Following the removal of the benzyloxycarbonyl group, N-tert-butyloxycarbonyl-S-benzyl-L-cysteine was incorporated in the form of its o-nitrophenyl ester.[14-16] The chain was then lengthened in the same manner by deprotection with trifluoroacetic acid and acylation with tert-butyloxycarbonyl amino acid o-nitrophenyl esters in each step until the protected nonapeptide, N-tert-butyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-homonorleucylglycinamide, was obtained. The high yields in the chain-lengthening steps demonstrated the usefulness of o-nitrophenyl esters as reactive derivatives in the stepwise synthesis[17] of polypeptide chains. The protected nonapeptide was treated with trifluoroacetic acid and the resulting trifluoroacetate salt converted to the free amine which in turn was reduced with sodium in liquid ammonia and cyclized by air oxidation. The crude cyclic peptide was purified by countercurrent distribution. In order to avoid the need for a separate removal of the N-protecting group, a second derivative of the same nonapeptide, N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-homonorleucylglycinamide, was also prepared, deblocked with sodium in liquid ammonia, and cyclized. An additional analog, 1-deamino-8-L-homonorleucine-vasopressin, was prepared by the method described for 1-deamino-oxytocin.[18,19] The remarkable insolubility of this compound prevented a study of its properties.

By rat vasopressor assay,[20] 8-L-homonorleucine-vasopressin has activity of 21.4 ± 1.0 units/mg or about 8% that of lysine-vasopressin. It has antidiuretic activity in ethanol-anesthetized rats[20] equal to about 10 units/mg as compared to about 250 units/mg for lysine-vasopressin.[21] These moderate effects, not very different from those reported for 8-formyllysine-vasopressin[2] or 8-leucine-vasopressin,[6,7] indicate that the amino group is not absolutely essential for the expression of vasopressin-like activities. They also show that a cation-forming group in the side chain of the eighth residue of vasopressin analogs is needed for potencies comparable to those of the natural hormone.§ A basic residue in this position is in itself not sufficient for high potency. This is shown by the example of 8-histidine-vasopressin.[22] An alternative replacement of the amino group by hydroxyl provides information about the possibility that the hormone is bound to the receptor, not through an ionic bond, but by a hydrogen bond. The corresponding analog, 8-L-hydroxynorleucine-vasopressin, was prepared in this laboratory.⁼ Its hormonal activities (30 units/mg of rat pressor and 70 units/mg of antidiuretic) are moderate but significantly higher than those of the homonorleucine analog, suggesting that a hydrogen bond is able to substitute, to some extent, for an ionic bond between hormone and receptor. Of course alternative explanations, such as polar interaction or hydrophilic effects, can also be invoked.

Experimental Section

Capillary melting points were taken and are uncorrected. Where analyses are indicated only by symbols of the elements, analytical results obtained for the elements were within ±0.4% of the theoretical values. For silica gel tlc, the following solvent systems were used: A, n-BuOH-AcOH-H_2O (4:1:1); B, $CHCl_3$-MeOH (9:1). Peptides were detected by the use of uv, ninhydrin.

† 1-Deamino-8-L-norleucine-oxytocin was prepared by M. Mühlemann, H. Nesvadba, and J. Rudinger, unpublished results (personal communication from Professor Rudinger).

‡ These experiments were carried out by Dr. Patricia Dreyfuss and Miss Basia Matthews.

§ The activities of the new vasopressin analogs were determined by Professor Wilbur H. Sawyer of the College of Physicians and Surgeons of Columbia University. The authors express their gratitude for this valuable contribution.

⁼ The synthesis of this analog was carried out by Dr. Patricia Dreyfuss and will be published separately.

chlorination, and charring techniques. For quantitative amino acid analysis, samples were hydrolyzed with constant boiling HCl in evacuated, sealed ampoules at 110° for 16 hr.

DL-Homonorleucine (**DL-Hnl**) was prepared according to the method published by Albertson.[8] The DL-amino acid was obtained by neutralization of the hydrochloride with triethylamine. A chromatographically homogeneous product was obtained, tlc R_f^A 0.45, mp 270–275° dec. *Anal.* ($C_7H_{15}N_1O_2$) C, H, N.

Acetyl-DL-Hnl was prepared by addition of acetic anhydride to DL-homonorleucine in boiling acetic acid as described by Greenstein and Winitz.[23] The product showed a single spot on tlc, R_f^A 0.59, mp 106–108°.

L-Hnl was obtained by action of hog renal acylase I on acetyl-DL-Hnl.[10] The product, mp 275° dec, was chromatographically homogeneous: tlc R_f^A 0.45; $[\alpha]^{22}D$ +23.1° (c 2, 5 *N* HCl) [lit.[24] +23.3° (c 2, 5 *N* HCl)].

Z-L-Hnl (I) was prepared from L-homonorleucine and benzyloxycarbonyl chloride using the procedure described for benzyloxycarbonylglycine.[25] The product was obtained in a yield of 94%: mp 63–65°; homogeneous on tlc, R_f^A 0.74; $[\alpha]^{22}D$ −3.5° (c 2, 95% EtOH). *Anal.* ($C_{15}H_{21}N_1O_4$) C, H, N.

Z-L-Hnl-Gly-OEt (**II**). Compound I (4.45 g, 15.9 mmol) was dissolved in a mixture of $CHCl_3$ (20 ml), toluene (20 ml), and triethylamine (TEA, 1.8 g) at room temperature and then cooled to −5°. Trimethylacetyl chloride (1.95 g, 15.9 mmol) was added and allowed to react for 1 hr at −5°. A precooled solution of glycine ethyl ester (2.25 g, 15.9 mmol) in chloroform (40 ml) with TEA (1.8 g) was added and the solution was allowed to come to room temperature. It was then washed with water, 0.5 *N* $KHCO_3$, and 0.5 *N* HCl. The organic layer was dried over $MgSO_4$, filtered, and evaporated. The residue was recrystallized from 50% ethanol: 84% yield; mp 109–110°; $[\alpha]^{22}D$ −15.6° (*c* 1.4, 95% EtOH); tlc R_f^B 0.22. *Anal.* ($C_{19}H_{28}N_2O_5$) C, H, N.

Z-L-Pro-L-Hnl-Gly-OEt (**III**). A solution of the protected dipeptide II (1.90 g, 5 mmol) in 95% EtOH was hydrogenated in the presence of an equimolar amount of HCl. The N-deprotected derivative was then acylated with Z-L-Pro-ONP[13] in DMF in the presence of TEA. The reaction was monitored by ninhydrin spot tests. The solvent was evaporated, the residue dissolved in chloroform, and the solution extracted with water, dried over $MgSO_4$, and evaporated. The crude product was triturated with ether. The solid which separated was collected and washed with ether to give 1.5 g (66%): mp 125–126°; $[\alpha]^{22}D$ −65.6° (*c* 2, 95% EtOH); chromatographically pure on tlc, R_f^B 0.63. An additional 0.2 g was recovered from the mother liquor, mp 126–128°. *Anal.* ($C_{24}H_{35}N_3O_6$) C, H, N.

Z-L-Pro-L-Hnl-Gly-NH₂ (**IV**). A solution of III (1.63 g, 3.6 mmol) in MeOH, which had been presaturated with NH_3 for 2 hr, was stoppered and left overnight. The MeOH was evaporated with a stream of nitrogen and the residue triturated with ether. The product was collected and washed with ether to give 1.46 g (94%) of a chromatographically homogeneous material: mp 164–165°; $[\alpha]^{22}D$ −59.8° (c 1, 95% EtOH); tlc R_f^B 0.32. *Anal.* ($C_{22}H_{32}N_4O_5$) C, H, N.

Boc-L-Cys(Bzl)-L-Pro-L-Hnl-Gly-NH₂ (**V**) was prepared from the tripeptide amide IV (1.59 g, 3.68 mmol). Deprotection was effected by HBr in AcOH and the chain was lengthened with a 20% excess of the active ester, Boc-Cys(Bzl)-ONO,[15] in DMF (12 ml) in the presence of diisopropylethylamine.[26] After completion of the reaction, determined by a ninhydrin spot test, the DMF was evaporated, water was added, and the product which separated was washed with ether: yield 1.83 g (84%); mp 164–165°; $[\alpha]^{22}D$ −43.9° (*c* 2, DMF containing 1% AcOH); tlc showed a single spot, R_f^B 0.48. *Anal.* ($C_{29}H_{45}N_5O_6S$) C, H, N, S.

Boc-L-Asn-L-Cys(Bzl)-L-Hnl-Gly-NH₂ (**VI**). Compound V (1.7 g, 2.88 mmol) was deprotected with trifluoroacetic acid (TFA). The trifluoroacetate was dissolved in DMF (12 ml) and allowed to react with Boc-Asn-ONO[15] in the presence of TEA. After completion of the reaction, EtOAc was added to the reaction mixture and the product separated. The product was collected and washed with DMF–EtOAc (v/v) and then with EtOAc: yield 1.5 g (75%); mp 214–215°; $[\alpha]^{22}D$ −59.0° (*c* 0.7, DMF containing 1% AcOH); tlc showed a single spot, R_f^A 0.71, R_f^B 0.29. Amino acid analysis gave Asp, 1.0; Pro, 1.1; Gly, 1.0; Hnl, 1.0; Cys(Bzl), 1.0. A second crop (0.25 g, mp 214–215°) was obtained from the mother liquor. *Anal.* ($C_{33}H_{51}N_7O_8S$) C, H, N, S.

Boc-L-Gln-L-Asn-L-Cys(Bzl)-LPro-L-Hnl-Gly-NH₂ (**VII**) was obtained from VI (0.74 g, 1.03 mmol) as described above using Boc-Gln-ONO[15] as the acylating agent: yield 0.79 g (92%); mp 209–210°; $[\alpha]^{22}D$ −45.1° (*c* 1, DMF containing 1% AcOH); tlc showed a single spot, R_f^A 0.69. Amino acid analysis gave Asp, 1.1; Glu, 1.0; Pro, 1.1; Gly, 1.0; Hnl, 0.9; Cys(Bzl), 0.9. *Anal.* Calcd for $C_{38}H_{59}N_9O_{10}S$: C, 54.7; H, 7.1; N, 15.1; S, 3.8. Found: C, 54.0; H, 7.2; N, 14.5; S, 4.1.

Boc-L-Phe-L-Gln-L-Asn-L-Cys(Bzl)-L-Pro-L-Hnl-Gly-NH₂ (**VIII**) was prepared from VII (0.69 g, 0.83 mmol) using a 100% excess of the acylating agent Boc-Phe-ONO[16] to prevent formation of the pyroglutamyl derivative. Reaction conditions were the same as described for VI: yield 0.71 g (88%); mp 224–225°; $[\alpha]^{22}D$ −43.2° (*c* 1, DMF containing 1% AcOH); tlc showed a single spot, R_f^A 0.55. Amino acid analysis gave Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; Phe, 1.0; Hnl, 1.0; Cys(Bzl), 0.9. *Anal.* ($C_{47}H_{68}N_{10}O_{11}S$) C, H, N, S.

Boc-L-Tyr(Bzl)-L-Phe-L-Gln-L-Asn-L-Cys(Bzl)-L-Pro-L-Hnl-Gly-NH₂ (**IX**). The protected octapeptide was prepared from VIII (0.69 g, 0.70 mmol) following the procedure described for VI, with Boc-Tyr(Bzl)-ONO[16] used for acylation: yield 0.83 g (96%); mp 226–228°; $[\alpha]^{22}D$ −36.6° (*c* 1, DMF containing 1% AcOH); tlc showed a single spot, R_f^A 0.59. Amino acid analysis gave Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; Tyr, 0.9; Phe, 1.0; Hnl, 1.0; Cys(Bzl), 0.9. *Anal.* ($C_{63}H_{83}N_{11}O_{13}S$) C, H, N, S.

Boc-L-Cys(Bzl)-L-Tyr(Bzl)-L-Phe-L-Gln-L-Asn-L-Cys(Bzl)-L-Pro-L-Hnl-Gly-NH₂ (**X**). Boc-Cys(Bzl)-ONO[15] was applied to lengthen the octapeptide chain (0.7 g, 0.57 mmol). The procedure used is described above, with the notable exception that diisopropylethylamine rather than Et_3N was used for the liberation of the free amine from its trifluoroacetate: yield 0.75 g (93%); mp 244–246°; $[\alpha]^{22}D$ −39.2° (*c* 1, DMF containing 1% AcOH); homogeneous on tlc, R_f^A 0.63. Amino acid analysis gave Asp, 1.1; Glu, 1.0; Pro, 1.0; Gly, 1.0; Tyr, 0.9; Phe, 1.0; Hnl, 1.1; Cys(Bzl), 2.1. *Anal.* ($C_{73}H_{94}N_{14}O_{14}S_2$) C, H, N, S.

L-Cys(Bzl)-L-Tyr(Bzl)-L-Phe-L-Gln-L-Asn-L-Cys(Bzl)-L-Pro-L-Hnl-Gly-NH₂ (**Xa**). The Boc group was removed from the protected nonapeptide X (0.23 g, 0.16 mmol) with TFA. The trifluoroacetate was dissolved in DMF and ten times the calculated amount of TEA was added to liberate the free amine which was then precipitated by dilution of the mixture with the EtOAc: yield 0.20 g (93%).

Z-L-Cys(Bzl)-L-Tyr(Bzl)-L-Phe-L-Gln-L-Asn-L-Cys(Bzl)-L-Pro-L-Hnl-Gly-NH₂ (**XI**) was prepared from compound IX (0.36 g, 0.3 mmol) as described for the corresponding *N-tert*-butyloxycarbonyl derivative. Z-L-Cys(Bzl)-ONP[13] was used for acylation and the product was obtained in a yield of 0.39 g (88%): mp 255–256°; $[\alpha]^{22}D$ −48.5° (c 0.4, AcOH); homogeneous on tlc, R_f^A 0.61. Amino acid analysis gave Asp, 1.0; Glu, 1.0; Pro, 0.9; Gly, 1.0; Tyr, 0.7; Phe, 0.9; Hnl, 1.0; Cys(Bzl), 1.8. *Anal.* ($C_{76}H_{92}N_{12}O_{14}S_2$) C, H, N, S.

***S*-Bzl-β-mercaptopropionyl-L-Tyr(Bzl)-L-Phe-L-Gln-L-Asn-L-Cys(Bzl)-L-Pro-L-Hnl-Gly-NH₂** (**XII**) was prepared from IX (0.3 g, 0.25 mmol) as described above using *S*-Bzl-β-mercaptopropionic acid *p*-nitrophenyl ester[18] as the acylating agent. The product, 0.28 g (85%), mp 248–250°, appeared homogeneous on tlc, R_f^A 0.6. Amino acid analysis gave Asp, 1.0; Glu, 1.0; Pro, 0.9; Gly, 1.0; Tyr, 0.8; Phe, 0.9; Hnl, 1.0; Cys(Bzl), 0.9. *Anal.* ($C_{68}H_{85}N_{11}O_{12}S_2 \cdot 2H_2O$) C, H, N.

8-L-Homonorleucine-vasopressin (**XIII**). **A** (**from X**). A sample (180 mg, 0.13 mmol) of the partially deprotected nonapeptide (Xa) was dissolved in liquid NH_3 (*ca.* 250 ml) at the boiling point of the solution, and small pieces of Na were added until the blue color persisted for about 3 min. After the addition of NH_4Cl (0.10 g), the NH_3 was allowed to evaporate to about 10 ml and the remaining solvent was removed *in vacuo* by evaporation from the frozen state. Dissolution of the residue in O_2-free water (200 ml) containing 0.08% AcOH was incomplete. Aeration was carried out at pH 6.5 for several hours until the sodium nitroferricyanide reaction became negative. The pH of the solution was then adjusted to 4 with AcOH; the solution was filtered and concentrated to a small volume *in vacuo*. As determined by uv absorption about two-thirds of the peptide was removed as insoluble material. Lyophilization was avoided because further polymerization was observed at previous attempts.

B (**from XI**). The benzyloxycarbonyl nonapeptide (XI, 50 mg) was reduced with Na in liquid NH_3 as described above, except that O_2-free 0.1% NH_4OH was used to dissolve the residue after evaporation of the NH_3. After air oxidation was complete, the solution was filtered and concentrated to a small volume. About 83% of the peptide was present in solution.

Several such concentrated preparations were pooled and, after filtration from insoluble material (polymer), subjected to countercurrent distribution in the system: *n*-BuOH–*n*-PrOH–0.05% AcOH (2:1:3). One major peak, detected by uv absorption at 278 nm, followed the curve calculated for K = 1.5, n = 468. The corresponding fractions were pooled and evaporated *in vacuo* to about 30 ml. A concentration of 1.0 mg/ml was determined by uv [λ_{max} 278 nm (ϵ 1400)] and quantitative amino acid analysis; $[\alpha]^{22}D$ −26.2° (*c* 0.1, 1 *N* AcOH); tlc showed a trace amount of an impurity and only a single strong spot $R_f{}^A$ 0.19, R_f 0.58 (*n*-PrOH-NH_4OH, 7:3). The minor spot might be due to polymerization of the product on the tlc plate itself. This tendency for polymerization interfered with further examinations by paper chromatography and electrophoresis. Amino acid analysis gave Asp, 1.0; Glu, 1.0; cystine, 1.0; Tyr, 0.9; Pro, 1.0; Hnl, 1.0; Gly, 1.0; Phe, 1.0.

1-Deamino-8-L-homonorleucine-vasopressin (XIV) was prepared from compound XII (0.22 g, 0.17 mmol) in the same manner as described for 8-L-homonorleucine-vasopressin. Although insoluble in O_2-free water containing 0.05% AcOH, the reduced material formed a disulfide linkage on air oxidation (determined by the nitroferricyanide test) in suspension. The product, collected by filtration (0.1 g, 58%), was found to dissolve readily in concentrated NH_4OH but not in H_2O: mp 253–254° dec; homogeneous on tlc, $R_f{}^5$ 0.39. Amino acid analysis gave Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; half-cystine, 0.87; mixed disulfide, 0.24; Tyr, 0.9; Phe, 0.9; Hnl, 1.0. *Anal.* ($C_{47}H_{65}N_{11}O_{12}S_2 \cdot H_2O$) C, H, N, S.

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Lipolytic Activity of Met-Arg-His-Phe-Arg-Trp-Gly, a Synthetic Analog of the ACTH (4–10) Core Sequence

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A new analog of the ACTH-(4–10)-heptapeptide has been synthesized by the solid-phase method. The thoroughly purified and characterized peptide, Met-Arg-His-Phe-Arg-Trp-Gly, was found to have about four times the lipolytic activity in isolated rabbit fat cells of the synthetic peptide, Met-Glu-His-Phe-Arg-Trp-Gly, corresponding to the natural sequence.

The heptapeptide sequence, Met-Glu-His-Phe-Arg-Trp-Gly, common to all the adrenocorticotropic (ACTH), melanotropic (MSH), and lipotropic (LPH) hormones yet investigated,[1,2] has been the subject of a large number of structure–function studies.[3-26] Of these seven amino acids, the pentapeptide sequence, His-Phe-Arg-Trp-Gly, positions 6–10 in ACTH, or even the position 6–9 tetrapeptide is either the minimal core of activity or is an essential element of the minimal core in all biological actions ascribed to any of these hormones.[1,3,27-31] The Met-Glu dipeptide sequence, positions 4 and 5 in ACTH, is not an absolutely essential element in most biological actions, but is always found in nature associated with the essential pentapeptide, and virtually always greatly enhances the minimal biological activity of the pentapeptide.[3,30,32-38] Several studies have produced data consistent with the hypothesis that the 4-methionine is involved in a hydrophobic interaction with the receptor,[12,13,15,20,22,26] and some of our recent work has suggested that the 5-glutamic acid may play a "spacer" role with little side-chain specificity.[39] We now wish to report that an analog of the heptapeptide sequence, in which the 5-glutamic acid is replaced by arginine, has about four times the activity of the natural sequence in the stimulation of free fatty-acid release in isolated rabbit fat cells.

Both the natural ACTH-(4–10)-peptide, methionylglutamylhistidylphenylalanylarginyltryptophylglycine, and the analog of interest, methionylarginylhistidylphenylalanylarginyltryptoptophylglycine, were synthesized by the solid-phase method.[40,41] These syntheses were performed on a Beckman Model 9900 peptide synthesizer, and the conditions for the two syntheses were virtually identical. Deprotection of the amino-protected intermediates on the resin was accomplished with 20% trifluoroacetic acid (TFA) in methylene chloride to which 5 mg/ml of dithiothreitol (DTT) had been added to protect against acid-catalyzed oxidations.[42] Double coupling of each amino acid was performed. The second coupling was followed by acetylation of any remaining free amino groups with acetylimidazole.[43] The protected peptide was