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Synthesis and Biological Activities of Arginine-vasopressin Analogues Designed from a Conformation–Activity Approach[1]

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Using the proposed conformation of vasopressin thought to be preferred when the mammalian antidiuretic hormone is bound to its renal receptor, an analogue, [1-β-mercaptopropionic acid,2-phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin, was designed that contained three synthetic modifications. It posessed a rat antidiuretic potency of 13 000 ± 1250 units/mg, no measurable rat pressor activity, an in vitro rat uterotonic potency of 6.0 ± 0.6 units/mg, and an avian vasodepressor potency of 2.9 ± 0.5 units/mg. The strong dissociation of an apparently very high antidiuretic activity of this analogue from its other biological activities prompted the synthesis of the singly and doubly modified analogues of this series of 3,4-dehydroproline-containing derivatives. [1-β-Mercaptopropionic acid,7-(3,4-dehydroproline)]arginine-vasopressin, [2-phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin, and [7-(3,4-dehydroproline)]arginine-vasopressin were found to have the following specific activities (units/mg), respectively: rat antidiuretic, 4134 ± 306, 1541 ± 336, 1260 ± 126; rat pressor, 240 ± 4.5, 63.8 ± 3.9, 255 ± 23; rat uterotonic, 69 ± 5, 0.80 ± 0.11, 40.6 + 4.7; avian vasodepressor, 345 ± 19, 4.2 ± 0.9, 76.9 ± 48. Using the same synthetic procedure, arginine-vasopressin was prepared in an overall yield of 51% with rat antidiuretic and pressor potencies of 511 ± 61 and 519 ± 13, respectively. Renal clearance studies revealed that arginine-vasopressin and [1-β-mercaptopropionic acid,2-phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin affect water and electrolyte handling by the kidney through a similar mechanism, but that the decrease in urine flow seen for the synthetic analogue is augmented by a decrease in glomerular filtration rate.

The conformation of a peptide hormone can serve as a model to design analogues in which a particular biological activity is maintained or enhanced while, at the same time, other biological activities of the parent hormone are diminished or even abolished.[2] Synthetic modifications that increase the capacity of "binding elements"[3] present in the hormone would be one way to accomplish this goal. Another way to enhance productive hormone–receptor interactions would be by modifications that increase the time the peptide spends in the "biologically active" conformation. One could postulate that a structural change reduces the inherent flexibility of the peptide backbone[4,5] or enhances compatibility beween the side chains of the analogue containing the binding elements and/or "active elements"[3] and the complementary receptor moieties.

For the vasopressins,

$$\overline{\text{Cys-Tyr-Phe-Gln-Asn-Cys}}\text{-Pro-}[^{\text{Arg}}_{\text{Lys}}]\text{-Gly-NH}_2$$

we proposed a model of the biologically active conformation thought to be favored for the elicitation of their antidiuretic activity.[6] In this model, amino acid side chains located at the corner positions of the two β turns (residues in positions 3, 4, 7, and 8) contain the binding elements (for schematic representation see Figure 1). The basic moiety of the position 8 residue and the carboxamide moiety of the asparagine residue in position 5 are the active elements. Using this model, we recently designed an analogue of vasopressin, [1-β-mercaptopropionic acid,2-phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin, ([β-Mpr^1,Phe^2,Δ^3-Pro^7]AVP) that contained three synthetic modifications and that possessed a remarkably high antidiuretic potency[4] (Table I). In this analogue the proline residue in position 7 of vasopressin was substituted by 3,4-dehydroproline, which has a double bond. The introduction of a deformable electron cloud was expected to enhance receptor binding of the resultant analogue, provided the steric fit at the receptor was correct.[7] The biologically active model of vasopressin predicts that the hydroxyl moiety on the tyrosine residue in position 2 is not an active element; hence, its deletion should not diminish the antidiuretic potency of the resultant Phe^2 analogue. The N-terminal amino group was replaced by a hydrogen atom. This deamination, which results in a tightening of the peptide backbone,[8] gives rise to vasopressin analogues that exhibit increased stability to enzymatic inactivation both in vitro and in vivo[9,10] (for a review see ref 11).

The enhancement of antidiuretic potency and specificity achieved with [β-Mpr^1,Phe^2,Δ^3-Pro^7]AVP prompted us to extend our biological testing of this analogue, as well as to synthesize and examine biologically the singly and doubly modified analogues of this series, which contain 3,4-dehydroproline in position 7. Accordingly, we synthesized [1-β-mercaptopropionic acid,7-(3,4-dehydroproline)]arginine-vasopressin ([β-Mpr^1,Δ^3-Pro^7]AVP),[12] [2-phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin ([Phe^2,Δ^3-Pro^7]AVP), and [7-(3,4-dehydroproline)]arginine-vasopressin ([Δ^3-Pro^7]AVP).[12]

The required protected peptide intermediates were prepared by the solid-phase technique,[13] using a chloromethylated polystyrene copolymer (1%)–divinylbenzene support to which glycine had been esterified, and a scheme of deprotection, neutralization, and coupling already described.[14] In general, the *tert*-butyloxycarbonyl (Boc) group was used for the temporary protection of the N$^\alpha$ group and was removed by treatment with 50% trifluoroacetic acid (TFA) in CH_2Cl_2. Coupling was affected by dicyclohexylcarbodiimide (DCC) or DCC mediated with 1-hydroxybenzotriazole (HBT),[15] except when indicated. The benzyl group was used for the protection of the

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Table I. Comparison of Biological Activities of Arginine-vasopressin with the Singly, Doubly, and Triply Modified Analogues[a]

peptide	antidiuretic (rat)	pressor (rat)	uterotonic (rat)	vasodepressor (fowl)
[β-Mpr¹,Phe²,Δ³-Pro⁷]AVP	13 000 ± 1250	nd[b]	6.0 ± 0.6	2.9 ± 0.5
[β-Mpr¹,Δ³-Pro⁷]AVP	4 134 ± 306	240 ± 4.5	69 ± 5	345 ± 19
[Phe²,Δ³-Pro⁷]AVP	1 541 ± 336	63.8 ± 3.9	0.80 ± 0.11	4.2 ± 0.9
[β-Mpr¹,Phe²]AVP[c]	810 ± 170	29 ± 7	0.3 ± 0.09	<1
[Δ³-Pro⁷]AVP	1 260 ± 126	255 ± 23	40.6 ± 4.7	76.9 ± 4.8
[β-Mpr¹]AVP[c]	1 400 ± 200	395 ± 20	29 ± 4	160 ± 4
[Phe²]AVP[d]	~350	122 ± 12	~0.2 ± 0.0	<1
AVP[e]	503 ± 53	487 ± 15	12.0 ± 0.2	100 ± 5
	511 ± 61[f]	519 ± 13[f]		

[a] Results are expressed in units/mg ± SEM and are based on the anhydrous weight of the peptide. [b] Potency values were not determined. See text for discussion. [c] See ref 20. [d] See ref 21. [e] See ref 22. [f] Potencies determined from a sample of AVP prepared by the procedure described herein.

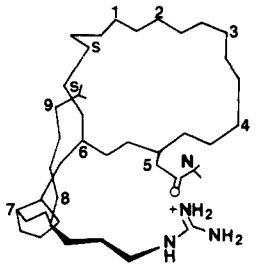

Figure 1. Active elements of the biologically active conformation shown in schematic representation of vasopressin (i.e., the side-chain carboxamide of Asn⁵ and the side-chain amino group of Arg⁸) for activation of the antidiuretic receptor. Side chains at the corner positions of the two β turns, Phe³, Gln⁴, Pro⁷, and Arg⁸ contain binding elements. Numbers indicate residue positions.

cysteine sulfhydryl groups and for the tyrosine hydroxyl group. The completed peptide was removed from the resin by ammonolysis in methanol.[16] Protecting groups, including the benzyloxycarbonyl (Z) group, when cysteine was the NH_2-terminal residue, were removed by treatment with sodium in anhydrous liquid NH_3.[17] The disulfide bond was formed by oxidation with 1,2-diiodoethane in aqueous methanol (1:1, v/v). The crude products were purified by gel filtration[18] and/or partition chromatography[19] on Sephadex.

Prior to the synthesis of the analogues, a synthesis of AVP was performed to establish the best synthetic protocol. It was found that in order to remove completely the Boc group from the arginyl residue a prewash with 50% TFA in CH_2Cl_2 was required. Otherwise, a proline deletion sequence impurity, which was extremely difficult to remove during purification, was present in the product. The protocol described under the Experimental Section gave crystalline protected AVP nonapeptide in 67% yield based on the original glycine content of the resin. A 76% yield was achieved for the conversion of the protected nonapeptide to AVP with rat pressor and antidiuretic potencies, matching the highest reported literature values (see Table I). Thus, the overall yield of AVP based in the glycine content of the resin was 51%. This yield matches or exceeds those obtained for the synthesis of oxytocin and vasopressin on benzhydrylamine-functionalized resin.[23]

Some of the pharmacological activities of the analogues were investigated and the results are shown in Table I. The antidiuretic and pressor potencies of [β-Mpr¹,-Phe²,Δ³-Pro⁷]AVP are highly dissociated. In Figure 2A note that a bolus injection of a 4.2×10^{-11} mol (21 microunits) dose of AVP caused a significant increase in

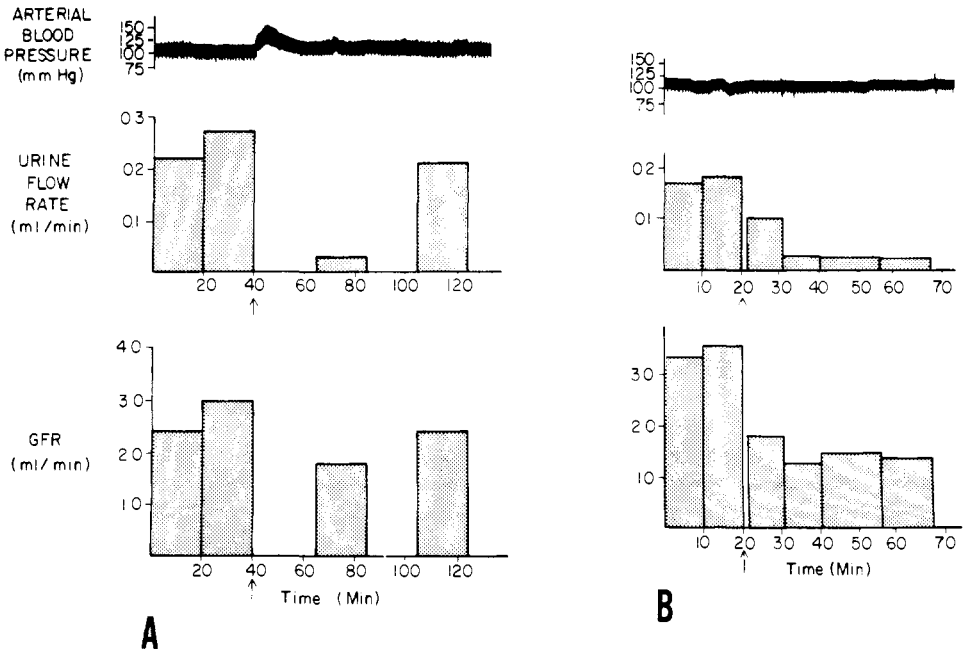

Figure 2. Typical effect on arterial blood pressure (mmHg), urine flow rate (mL/min), and glomerular filtration rate (mL/min) in hydrated rat. Panel A: single intravenous bolus injection of 4.2×10^{-11} mol (21 microunits) of arginine-vasopressin. Panel B: single intravenous bolus injection of 1.6×10^{-12} mol (21 microunits) of [β-Mpr¹,Phe²,Δ³-Pro⁷]AVP.

blood pressure. On the other hand, Figure 2B shows that an equipotent antidiuretic dose (1.6×10^{-12} mol, 21 microunits) of [β-Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP was without pressor effect. In fact, to obtain even a minimal detectable pressor response a dose of 30 000 times as large as the effective antidiuretic dose of [β-Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP was needed. The antidiuretic potency of the analogues was measured by the intensity of the antidiuretic response, as determined from the maximal depressions of urine flow following intravenous injections. This method has been reported to yield values least subject to errors caused by differing durations of antidiuretic responses of analogue and standard.[24] In fact, the response patterns to equipotent doses of [β-Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP, [β-Mpr[1],Δ[3]-Pro[7]]AVP, and AVP were nearly identical. In addition, the potencies of these two highly active analogues determined in rats of the Brattleboro strain, homozygous for diabetes insipidus, were the same as those obtained with Sprague–Dawley rats, indicating that the response is due to direct action on the kidney and not to release of endogenous vasopressin. [β-Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP did not cause autopotentiation nor autoattenuation when a series of five 1.5×10^{-12} mol doses were administered, preceeded and followed by a 21-microunit dose of AVP.

The extraordinarily high antidiuretic potency of [β-Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP prompted us to investigate further the mechanism of its antidiuretic activity. Renal clearance studies showed that intravenous injection of 21-microunits of AVP or [β-Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP produced similar effects in decreasing urine flow (Figure 2A,B) and fractional excretion of sodium (from 0.012 to 0.006), increasing urine osmolality (from 200 to 700 mosm/L), and reversing free-water clearance by the same magnitude. At this low dose, the glomerular filtration rate (GFR) decreased with administration of the analogue (Figure 2B), whereas GFR changed little with AVP (Figure 2A). The effect on water and electrolyte handling by the kidney leading to antidiuresis appears to be through a similar mechanism for the hormone and the analogue, but the decrease in urine flow seen for [β-Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP is augmented by its effect on GFR.

An explanation of the effect on GFR by [β-Mpr[1],-Phe[2],Δ[3]-Pro[7]]AVP is possible when the effect of AVP on glomerular microcirculation is taken into account. Ichikawa and Brenner[25] have shown that AVP decreases the glomerular ultrafiltration coefficient (K_f). At the same time the glomerular transcapillary hydraulic pressure difference (ΔP) increases, primarily due to a decrease in Bowman's space hydraulic pressure. These compensatory changes give rise to little or no change in GFR. One only need imagine the case of a hormone analogue whose potency at the glomerular filtration membrane in decreasing K_f is greater than can be offset by the maximum decrease of the back pressure in Bowman's space. In such a case, the GFR would be expected to fall.

The complex nature of the mechanism of action of vasopressin in the kidney, combined with the possibility that vasopressin analogues may interact preferentially with different vasopressin-sensitive receptors in the nephron (e.g., in the glomerulus,[26] ascending portion of the loop of Henle,[27] or in the collecting duct[27]), requires that parameters of renal response, in addition to urine flow rate, be measured in order to describe accurately the action of synthetic vasopressin analogues on the kidney.

Experimental Section

Melting points were determined in open capillary tubes and are reported uncorrected. Optical rotations were measured in a Zeiss Circle polarimeter at 0.01°. For thin-layer chromatography, loads of 50 μg were applied to precoated plates of silica gel 60 F-254 (E. Merck, Darmstadt). Chromatograms were developed for 100–150 mm in the following solvent systems (all by volume): (A) 1-BuOH–HOAc–H_2O (4:1:1); (B) 1-BuOH–pyridine–HOAc–H_2O (15:10:3:6); (C) 1-PrOH–HOAc–H_2O (3:1:1); (D) HOAc–$CHCl_3$–CF_3CH_2OH (1:8:8); (E) EtOAc–pyridine–HOAc–H_2O (5:5:1:3). Visualization was made with Cl_2, followed by 4,4-bis(tetramethyldiamino)diphenylmethane/KI spray[28] or by starch–KI (1:1%) spray. Amino acid analysis[29] was performed on a Durrum-Dionex D-500 amino acid analyzer, following hydrolysis for 24 h in constant-boiling HCl at 110 °C in vacuo. A duplicate hydrolysis included phenol, 0.2%, when Tyr was present.[30] When elemental analyses appear only as the symbols of the elements, data fall within ±0.4% of the theoretical value.

Rat uterine assays were performed on isolated horns from virgin rats in natural estrus according to the method of Holton,[31] as modified by Munsick,[32] with the use of Mg^{2+}-free van Dyke–Hastings solution as bathing fluid. Avian vasodepressor assays were performed on conscious chickens by the method of Coon,[33] as described in the U.S. Pharmacopeia,[34] as modified by Munsick et al.[35] Pressor assays were carried out on anesthetized male rats as described in the U.S. Pharmacopeia.[36] Antidiuretic assays were performed on anesthetized male rats according to the method of Jeffers et al.,[37] as modified by Sawyer.[38] The four-point assay design of Schild[39] was used to obtain specific activities as compared to U.S.P. posterior pituitary reference standard.

Renal clearance experiments were performed on six male Sprague–Dawley rats weighing 250–275 g. They were anesthetized with Inactin (10 mg/100 g of body weight) given intraperitoneally. All animals were hydrated to 8% body weight with a solution of 2% ethanol and 0.5% glucose. Tracheotomy was performed, and one carotid artery and one jugular vein were cannulated. The bladder was catheterized via an abdominal incision. Blood pressure was obtained via the cannulation of the carotid artery and recorded on a two-channel physiograph. Body temperature, determined rectally, was maintained at 37 °C throughout the experiment. At the start of the experiment, an infusion solution of 3% inulin, 1% mannitol, 0.6% NaCl, and 2% alcohol (300 mosm) was infused at a constant rate of approximately 10 mL/h. After an equilibration period of 60 min, urine samples were collected under mineral oil in preweighed glass vials, and the urine volume was determined gravimetrically. Blood samples were collected during the midportion of each clearance period. Two control periods were attempted to provide a basis upon which to compare the postinfusion variables. Then, either AVP at 4.2×10^{-11} mol or [Mpr[1],Phe[2],Δ[3]-Pro[7]]AVP at 1.6×10^{-12} mol was injected intravenously, and blood and urine collections were continued as before. A total of four postinjection samples were attempted. The concentration of inulin in plasma and urine was determined by an anthrone method[40] from which GFR was calculated. Plasma and urinary sodium and potassium concentrations were estimated using standard flame photometry.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg-(Tos)-Gly-NH_2 (1). To a polystyrene copolymer–1% divinylbenzene (Lab Systems, Inc., 0.75 mmol/g substitution as Cl), Boc-Gly was attached according to the method of Gisin.[41] Following hydrolysis for 24 h in propionic acid–HCl (1:1) at 132 °C in vacuo, amino acid analysis yielded 0.57 mmol of Gly/g of resin. Standard solid-phase techniques[13] as previously described[14] were employed throughout the synthesis using 10 g (5.7 mmol) of Boc-Gly-*O*-resin with a solvent volume of 120 mL per step. Removal of the Boc group was accomplished in 50% CF_3CO_2H in CH_2Cl_2, containing 2% anisole. Couplings were performed with 2.5 equiv of *tert*-butyloxycarbonyl-protected amino acid and DCC in CH_2Cl_2 for 2 h. When HBT[15] was included in the coupling mixture, 5 equiv was employed. Repeat couplings, when performed, used 0.5 equiv of *tert*-butyloxycarbonyl-protected amino acid and DCC and 1 equiv of HBT. For all HBT-mediated couplings, *tert*-butyloxycarbonyl-protected amino acid and HBT were added in dimethylformamide (DMF), followed by DCC in CH_2Cl_2. HBT-mediated coupling mixtures were agitated for 4–12 h, with the addition of 1 equiv of diisopropylethylamine (*i*-Pr_2NEt) after 2 h. A coupling was judged complete (better than 99.4%) by a ninhydrin-negative test.[42] Boc-Arg(Tos) (10% DMF added to CH_2Cl_2 to affect solution), Boc-Pro, and Boc-Cys(Bzl) were all successfully attached after a single 2-h DCC coupling. The

remaining residues were coupled with a single 4-h HBT-mediated DCC coupling to which 1 equiv of $(i\text{-Pr})_2$NEt was added after 2 h. The peptide resin was finally washed with CH_2Cl_2 and dried at 40 °C in vacuo for 3 days: yield 18.7 g (95% of theoretical weight gain based on Gly content of resin). The peptide resin was suspended in anhydrous CH_3OH [distilled from $Mg(OCH_3)_2$], saturated with NH_3 at 0 °C, and stirred in a sealed container for 4 days. The solvents were evaporated and the peptide was extracted into warm (60 °C) DMF (three times, 135 mL). To the combined extracts (kept at 60–80 °C) was added 150 mL of boiling H_2O. As the solution was cooled and stirred, the product precipitated. The precipitate was filtered, washed with H_2O–DMF (1:1, 200 mL), H_2O (three times, 200 mL), 95% EtOH (twice, 200 mL), Et_2O (twice, 200 mL), and dried in vacuo: yield 6.35 g, mp 222–224 °C. The precipitate was crystallized from HOAc–EtOH as described for another protected nonapeptide of AVP:[43] yield 6.31 g (67% yield based on Gly content of resin); mp 226–228 °C $[\alpha]^{26}_D$ −14° (*c* 1.0, DMF); TLC (A) R_f 0.58; (B) R_f 0.68. Amino acid analysis: Asp, 0.97; Glu, 1.02; Pro 0.99; Gly, 1.00; Tyr, 0.92; Phe, 1.03; Cys(Bzl), 1.76; NH_3, 3.04; Arg, 0.95.

Arginine-vasopressin (2). The protecting groups were removed from 1 (329 mg, 0.2 mmol) by treatment with Na in refluxing anhydrous liquid NH_3 (125 mL). After 30 s, the blue color of excess Na was discharged with 3 drops of glacial HOAc and the NH_3 removed by evaporation under a stream of N_2. The residue was dissolved in CH_3OH–H_2O (180:200 mL, precooled to 4 °C), and to the solution was added ICH_2CH_2I (59 mg, 0.2 mmol) in CH_3OH (10 mL). The disappearance of sulfhydryl was monitored by the method of Ellman.[44] The reaction was complete after 10 min, HOAc (5 mL) was added, and the volume was reduced to 3 mL. To this, HOAc (3 mL) was added and the solution applied to a 2.15 × 118 cm column of Sephadex G-15 (fine), which had been preequilibrated with 50% HOAc. The column was eluted with 50% HOAc at 8.4 mL/h and the eluate collected in 2.3-mL fractions. Peptide material was detected by monitoring the absorbancy at 280 nm. A minor peak appeared at 124 mL (31% of column volume), followed by a major peak at 170 mL (43%) of the column volume. The fractions containing the major peak were pooled; the pooled volume was reduced to 5 mL, diluted with 0.2 N HOAc (50 mL), and reduced again to 5 mL. The product was further purified by gel filtration on a 2.15 × 118 cm column of Sephadex G-15 (fine) which had been preequilibrated with 0.2 N HOAc. The column was eluted with 0.2 N HOAc, and 3.65-mL fractions were collected at 10.3 mL/h. The product emerged as a single, slightly tailing peak, with a maximum at 259 mL (60% column volume). TLC was performed on 1–3-μL aliquots of each fraction across the peak in system B; all fractions revealed a major spot, R_f 0.36; two minor impurities were detected, R_f 0.32 and 0.43 (eluting from 252–256 mL). Fractions containing the homogeneous product (259–288 mL) were pooled, carefully frozen at −20 to −30 °C, and lyophylized from 0.2 N HOAc: yield 164 mg (76% from nonapeptide corrected to anhydrous weight); TLC (B) R_f 0.36; (C) R_f 0.13; (E) R_f 0.89. Amino acid analysis: $Cys(O_3H)$,[45] 2.01; Asp, 1.01; Glu, 1.04; Pro, 1.04; Gly, 1.00; Tyr, 0.96; Phe, 1.02; NH_3, 3.00; Arg, 0.99.

Boc-Phe-Gln-Asn-Cys(Bzl)-Δ³-Pro-Arg(Tos)-Gly-*O*-resin (3). A 5-g portion of the same Boc-Gly-*O*-resin (2.85 mmol) used for the synthesis of 1 was employed with a solvent volume of 80 mL per step. The same procedure as described for 1 was followed but with each residue double coupled. The exceptions were Δ³-Pro,[46] which was attached in a single overnight coupling using only 1.5 equiv of Boc-Δ³-Pro, 2.5 equiv of DCC, and 3 equiv of HBT, and the glutamyl residue, which was coupled as Boc-Gln-ONp. The heptapeptide resin was washed in methanol and dried in vacuo at 40 °C: yield 7.4 g (86% from Boc-Gly-*O*-resin). Amino acid analysis following hydrolysis for 24 h at 130 °C in propionic acid–HCl (1:1) in vacuo gave the following values in mmol/g of heptapeptide–resin (molar ratios in parentheses, based on Gly): Asp, 0.35 (0.91); Δ³-Pro,[47] 0.31 (0.80); Glu, 0.37 (0.95); Gly, 0.39 (1.00); Phe, 0.32 (0.84); Cys(Bzl), 0.31 (0.80); NH_3, 0.74 (1.92); Arg, 0.33 (0.85). This synthesis was repeated using another 5 g of the same Boc-Gly-*O*-resin with identical results.

β-Mpr(Bzl)-Phe-Phe-Gln-Asn-Cys(Bzl)-Δ³-Pro-Arg(Tos)-Gly-NH_2 (4). A 7.1-g portion of 3 was elongated with a single HBT-mediated coupling of Boc-Phe to yield, after drying in vacuo at 40 °C, 7.5 g of octapeptide–resin. β-Mpr(Bzl)[48] was attached to 3.76 g of the octapeptide resin with a single HBT-mediated coupling and using 40-mL volumes per step. The protected peptide was cleaved from the resin by ammonolysis and purified as described for 1 to give an amorphous powder: yield 1.08 g (55% based on Gly content of resin); mp 218–220 °C dec; $[\alpha]^{28}_D$ −68° (*c* 1.0, DMF); TLC (A) R_f 0.48 with a detectale impurity at R_f 0.43. Amino acid analysis: Asp, 1.00; Δ³-Pro, 0.97; Glu, 1.04; Gly, 1.00; Phe, 1.94; Cys(Bzl), 0.94; NH_3, 2.91; Arg, 0.97. Anal. ($C_{67}H_{82}N_{14}O_{13}S_3 \cdot H_2O$) C, H, N.

[1-β-Mercaptopropionic acid,2-phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin (5). A portion of 4 (208 mg, 0.148 mmol) was treated with sodium in liquid NH_3, the cyclic disulfide bond was formed and the product was partially purified by gel filtration on Sephadex G-15 (fine) as described for 2, except that the peptide material was detected by monitoring the column eluate at 257.5 nm. Fractions containing the major peak were pooled, diluted with H_2O, and evaporated twice to a volume of 2 mL by rotary evaporation. The upper phase (2 mL) of the solvent system 1-BuOH–EtOH–pyridine–0.2 N HOAc (6:1:1:8) was added to the aqueous solution, and the sample was applied to a 2.82 × 65 cm column of Sephadex G-25 (100–200 mesh, block polymerizate) which had been equilibrated with the lower phase for purification by partition chromatography.[19] The column was eluted with the upper phase and collected in 2.8-mL fractions. Peptide material was detected by the method of Lowry in 0.1-mL aliquots withdrawn from every third tube.[49] The product emerged as a single peak with a maximum at R_f 0.47. TLC in system B across the peak revealed a major product at R_f 0.49 and an impurity at R_f 0.52 concentrated in the tubes of the leading edge. Fractions were chosen so as to maximize purity but not yield, and the product was subjected to another partition chromatography in the same system, on the same column, but with the column having been equilibrated with both phases of the solvent system. This time the product emerged on a sharp peak with a small partially overlapping leading peak. Only those fractions which were homogeneous by TLC were pooled and diluted with H_2O; the organic phase was removed by evaporation and the aqueous solution was lyophilized: yield 91.1 mg (53% yield from 4); $[\alpha]^{24}_D$ −148° (*c* 0.44, 1 M HOAc); TLC (A) R_f 0.40, (B) R_f 0.49. Amino acid analysis: $Cys(O_3H)$, 0.98; Asp, 1.00; Δ³-Pro, 1.00; Glu, 1.02; Gly, 1.00, $^1/_2$-Cys, 0.43; mixed disulfide of β-Mpr-Cys, 0.48; Phe, 1.97; NH_3, 2.81; Arg, 1.00. Anal. ($C_{46}H_{63}N_{14}O_{11}S_2 \cdot CH_3CO_2H \cdot 3H_2O$) C, H, N.

Z-Cys(Bzl)-Phe-Phe-Gln-Asn-Cys(Bzl)-Δ³-Pro-Arg(Tos)-Gly-NH_2 (6). Beginning with 3.75 g of the octapeptide resin prepared for 4, Z-Cys(Bzl) was successfully coupled with a single overnight DCC–HBT coupling. After drying at 40 °C in vacuo, the peptide resin weighed 3.81 g. The protected nonapeptide was cleaved from the resin by ammonolysis as described for 1, and the product was purified by precipitation from DMF with H_2O. The precipitate was filtered; washed with H_2O, EtOH, and Et_2O; and dried in vacuo: yield 1.45 g (66% from Boc-Gly-*O*-resin); mp 219–221 °C dec; $[\alpha]^{28}_D$ −55° (*c* 1.0, DMF); TLC (D) R_f 0.32 with one trace impurity, R_f 0.48. Amino acid analysis: Asp, 0.99; Δ³-Pro, 0.97; Glu, 1.05; Gly, 1.00; Phe, 1.97; Cys(Bzl), 1.88; NH_3, 2.90; Arg, 0.96. Anal. ($C_{75}H_{89}N_{15}S_3 \cdot H_2O$) C, H, N.

[2-Phenylalanine,7-(3,4-dehydroproline)]arginine-vasopressin (7). The protecting groups from 6 (231 mg, 0.49 mmol) were removed by treatment with sodium in liquid NH_3, the cyclic disulfide bond was formed, and the product was partially purified by two gel-filtration steps on a Sephadex G-15 (fine) column in 50% HOAc and 0.2 N HOAc as described for 2. The product was further purified by a single partition chromatography in the system 1-BuOH–EtOH–pyridine–0.1 N HOAc (4:1:1:7) as described for 5. The product emerged as a single peak with a maximum at R_f 0.33. Fractions comprising the peak were pooled, and the product was isolated by lyophilization as described for 5: yield 81.5 mg (64% from 6); $[\alpha]^{25}_D$ −59° (*c* 0.5, 1 N HOAc); TLC (B) R_f 0.38, (C) R_f 0.15, (E) R_f 0.88. Amino acid analysis: $Cys(O_3H)$, 2.02; Asp, 1.01; Δ³-Pro, 0.95; Glu, 1.03; Gly, 1.00; Phe, 2.06; NH_3, 3.02; Arg, 1.00. Anal. ($C_{46}H_{65}N_{15}O_{11}S_2 \cdot 2CH_3CO_2H \cdot H_2O$) C, N; H: calcd 6.27; found, 5.72.

β-Mpr(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Δ³-ProArg(Tos)-Gly-NH_2 (8). Beginning with 7.2 g of 3, Boc-Tyr(Bzl) was coupled with a single DCC–HBT coupling. The

octapeptide resin was divided into two equal parts, and one part was extended by double coupling β-Mpr(Bzl) onto it. The protected peptide was cleaved from the resin by ammonolysis, and the product was purified as described for 1: yield 1.05 g (51% from Boc-Gly-*O*-resin); mp 212–215 °C dec; $[\alpha]^{27}_D$ −53° (*c* 1.00, DMF); TLC (A) R_f 0.47 with two impurities, R_f 0.42 and 0.52. Amino acid analysis: Asp, 1.01; Δ^3-Pro, 0.98; Glu, 1.02; Gly, 1.00; Tyr, 0.91; Phe, 0.98; Cys(Bzl), 0.93; NH_3, 2.88; Arg, 0.97. Anal. ($C_{74}H_{88}N_{14}O_{14}S_3 \cdot H_2O$) C, H, N.

[1-β-Mercaptopropionic acid,7-(3,4-dehydroproline)]arginine-vasopressin (9). The protecting groups from 8 (224 mg, 0.148 mmol) were removed by treatment with sodium in liquid NH_3, the cyclic disulfide bond was formed, and the product was partially purified by gel filtration on Sephadex G-15 (fine) in 50% HOAc as described for 2. The product was further purified by partition chromatography in the system 1-BuOH–EtOH–pyridine–0.2 N HOAc (6:1:1:8) as described for 5, emerging with an R_f of 0.30. The product was finally subjected to gel filtration on a 2.82 × 68 cm column of Sephadex G-25 (block polymerizate, 200–270 mesh) in 0.2 N HOAc, emerging at 88% of the column volume. The fractions comprising the peak area were pooled and lyophilized: yield 76.4 mg (41% yield from 8); $[\alpha]^{24}_D$ −159° (*c* 0.5, 1 M HOAc); TLC (A) R_f 0.46, (C) R_f 0.38. Amino acid analysis: Cys(O_3H), 1.00; Asp, 1.01; Δ^3-Pro, 0.94; Glu, 1.03; Gly, 1.00; $^1/_2$-Cys, 0.43; mixed disulfide of Cys and β-Mpr, 0.41; Tyr, 0.98; Phe, 0.99; NH_3, 3.00; Arg, 1.00. Anal. ($C_{46}H_{63}N_{14}O_{12}S_2 \cdot 2CH_3CO_2H \cdot 3H_2O$) C, H, N.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Δ^3-Pro-Arg(Tos)-Gly-NH_2 (10). The remaining portion of the octapeptide resin prepared for 8 was extended by a single DCC–HBT coupling of Z-Cys(Bzl). The protected nonapeptide was cleaved from the resin as described for 1, and the product was purified by precipitation from DMF with H_2O. The precipitate was filtered; washed with H_2O, EtOH, and Et_2O; and dried in vacuo: yield 1.43 g (61% from Boc-Gly-*O*-resin); mp 221–223 °C dec; $[\alpha]^{28}_D$ −66° (*c* 1.0, DMF); TLC (D) R_f 0.37, with a trace impurity at R_f 0.12. Amino acid analysis: Asp, 1.00; Δ^3-Pro, 0.97; Glu, 1.05; Gly, 1.00; Tyr, 0.87; Phe, 1.00; Cys(Bzl), 1.87; NH_3, 3.04; Arg, 0.97. Anal. ($C_{82}H_{95}N_{15}O_{16}S_3 \cdot CH_3CO_2H$) C, H, N.

[7-(3,4-Dehydroproline)]arginine-vasopressin (11). The protecting groups from 10 (245 mg, 0.144 mmol) were removed by treatment with sodium in liquid NH_3, the cyclic disulfide bond was formed, and the product was purified by gel filtration on columns of Sephadex G-15 and isolated by lyophilization described for 2: yield 135.8 mg (75% from 2); $[\alpha]^{25}_D$ −63° (*c* 0.42, 1 M HOAc); TLC (B) R_f 0.42, (C) R_f 0.15, (E) R_f 0.85. Amino acid analysis: Cys(O_3H), 1.90; Asp, 0.99; Δ^3-Pro, 0.95; Glu, 1.02; Gly, 1.00; Tyr, 0.90; Phe, 1.00; NH_3, 3.03; Arg, 0.99. Anal. ($C_{46}H_{65}N_{15}O_{12}S_2 \cdot 2.5CH_3CO_2H \cdot H_2O$) C, H, N.

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References and Notes

(1) Abbreviations follow the IUPAC–IUB Tentative Rules on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations are: β-Mpr, β-mercaptopropionic acid; HBT, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; Δ^3-Pro, 3,4-dehydroproline; TFA, trifluoroacetic acid; DMF, dimethylformamide; *i*-Pr_2NEt, diisopropylethylamine; AVP, arginine-vasopressin; GFR, glomerular filtration rate; K_f, glomerular ultrafiltration coefficient; ΔP, glomerular transcapillary hydraulic pressure difference. Optically active amino acids are of the L configuration.
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Tripeptide Analogues of Melanocyte-Stimulating Hormone Release-Inhibiting Hormone (Pro-Leu-Gly-NH_2) as Inhibitors of Oxotremorine-Induced Tremor

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Fourteen di- and tripeptide analogues of MIF, Pro-Leu-Gly-NH_2, have been synthesized and assayed for inhibition of oxotremorine-induced tremor. Replacement of Pro by HCO-Pro or cyclopentanecarboxylic acid gave inactive analogues, while some peptides of the general structure <Glu-Leu-Gly-NR_1R_2 were highly active. Thus, $R_1 = C_3H_8$ and R_2 = H gave 4 times the activity of MIF, $R_1 = i\text{-}C_3H_8$ and R_2 = H gave 13 times the activity of MIF, and $R_1 = R_2 = CH_3$ gave 29 times the activity of MIF. *cyclo*(-Pro-Leu-), Pro-Lys-Gly-NH_2, and Pro-Arg-Gly-NH_2 had no activity. Apparently, small modifications in the structure of MIF can yield highly active analogues with potential clinical value, e.g., in the treatment of Parkinson's disease or mental depression.

Prolylleucylglycine amide (MSH release-inhibiting hormone, MIF, melanostatin) is a peptide with pronounced direct effects on the central nervous system. Several studies have shown that MIF potentiates the behavioral effects of L-Dopa[1,2] or apomorphine,[3] reverses the central and peripheral effects of oxotremorine,[4,5] antagonizes the sedative effects of deserpidine,[6] attenuates puromycin-induced amnesia,[7] facilitates morphine dependence,[8] and influences learning and spontaneous behavior in several species.[3,9-11] The first four effects are typical for drugs active against Parkinson's disease. In agreement with these data, MIF has been shown to ameliorate the symptoms of clinical parkinsonism,[12-14] either alone or in conjunction with L-Dopa. Increasing experimental evidence also points to a beneficial effect of MIF in mental depression.[15-17]

However, large doses of peptide are required to bring about clinical effects, which has prompted several structure–activity studies on MIF in the search for more active analogues.[18-23] Evaluation of relative activities has mainly been performed by means of an oxotremorine-antagonism test.[18-21] Evidently, the structural requirements for activity are very strict. Only analogues retaining the original tripeptide amide backbone have shown any tremorolytic effect, while tetrapeptides and dipeptides (potential metabolites) related to MIF are inactive.[19] Replacement of the pyrrolidine ring of Pro by a cyclopentane (Cpc) or thiazolidine (Thz) ring causes loss of activity,[21] while substitution of <Glu for Pro even increases the tremorolytic effect.[18]

Modification of the primary amide of Gly generally causes loss of activity,[19-21] the important exception being the highly active <Glu-Leu-Gly-NH-C_2H_5.[19] The lipophilic ethyl group could conceivably facilitate the diffusion of the analogue to its target site, since no mechanism for active uptake of MIF seems to exist.[24] Replacement of Gly-NH_2 by Pro-NH_2 gives some retention of oxotremorine antagonism, while substitution of DL-thiazolidine-2-carboxamide for Gly-NH_2 causes loss of activity in this test but retention of activity in the Dopa potentiation test.[21]

A few analogues have been evaluated as inhibitors of fluphenazine-induced catalepsy.[23] MIF itself was in this test active only after chronic administration, while analogues in which *N*-Me-Leu (D or L) had been substituted for Leu were active after a single injection. In the Dopa potentiation test they were not as potent as MIF.

From the data in the cited literature it is clear that high activity in one test system does not necessarily correlate with high activity in another. Whether any of these systems correlates well with clinical efficiency remains to be established. The picture is further complicated by the observation that very often MIF is active only within a narrow dose range.[25]

The present study explores some possible ways of developing highly active MIF analogues.

Results and Discussion

Chemistry. Peptides **4–8** were prepared from Z-Leu-Gly-OEt (**1**). Acidolysis of **1** and coupling with HCO-Pro[26] (DCI/HOBT) or Cpc-OPcp[27] gave HCO-Pro-Leu-Gly-OEt (**3**) and Cpc-Leu-Gly-OEt (**6**), respectively. Aminolysis with NH_3 or $EtNH_2$ gave the analogues **4**, **5**, **7**, and **8**.

Analogues **13–16**, **20**, **21**, and **25** were prepared from Z-<Glu-Leu (**10**). An attempted direct coupling of Z-<Glu-OPcp and the sodium salt of Leu in dioxane–H_2O (2:1) at pH 11 gave **10** in a 33% yield and 34% of the byproduct Z-Glu-Leu, which accounts for the consumption of 1 equiv of NaOH. Synthesis of **10** by way of the intermediate Z-<Glu-Leu-OBu^t (**9**) was more successful, since the *tert*-butyl ester could be selectively removed with $BF_3 \cdot Et_2O$ in HOAc, giving **10** in an overall yield of 64%. From **10**, <Glu-Leu-Gly-OEt (**12**) was prepared by a DCI/HOBT-mediated condensation with Gly-OEt·HCl

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