Photoaffinity, Biotinyl, and Iodo Analogues as Probes for Vasotocin Receptors†

Angeliki Buku, Diana Gazis, and Patrick Eggena*

Department of Physiology and Biophysics, Mount Sinai Medical and Graduate School, City University of New York, New York, New York 10029. *Received January 25, 1989*

Vasotocin (AVT) analogues with tyrosine or phenylalanine in position 9, i.e., [9-tyrosine]AVT, [2-phenylalanine,-9-tyrosine]AVT, and 1-desamino[9-(*p*-aminophenylalanine)]AVT, were synthesized by the solid-phase method. These compounds showed a high biological activity in the hydroosmotic toad bladder assay. Using the chemically reactive functional groups on tyrosine and *p*-aminophenylalanine in position 9, we prepared iodinated, photoreactive, and affinity ligands, i.e., [2-phenylalanine,9-(iodotyrosine)]AVT, 1-desamino[9-(*p*-azidophenylalanine)]AVT, and 1-desamino[9-(biotinylphenylalanine)]AVT, respectively. Half-maximal hydroosmotic responses (ED-50 values) were obtained with 2.5×10^{-9} M for the iodinated analogue, with 0.9×10^{-10} M for the photoaffinity analogue, and with 1.2×10^{-8} M for the biotinyl analogue. The hydroosmotic activity of the biotinyl analogue was reversed following addition of avidin, whereas the photoaffinity analogue induced a persistent response following UV irradiation that was not reversed upon repeated and prolonged periods of washout. These analogues of vasotocin are the most potent that have been synthesized to date, and they should serve as useful probes in the isolation and characterization of vasotocin receptors in toad bladders and tissues from other species that use vasotocin as an antidiuretic/pressor principle. The photoaffinity and biotinyl analogues had a rat antidiuretic activity of 66 and 40 units/mg, respectively. These compounds are, therefore, also suitable for the isolation of V-2 vasopressin receptors from mammalian tissues.

The antidiuretic hormone employed by fish, birds, reptiles, and amphibians, including the toad, is [8-arginine]-vasotocin (AVT).[1,2] In the toad, vasotocin increases the permeability to water of the urinary bladder.[3] The mammalian antidiuretic hormone, vasopressin, acts similarly on the bladder, although about 50-fold higher concentrations are required.[4] When the bladder is stimulated with photosensitive analogues of vasopressin in the presence of long-wavelength UV light, the analogues form covalent complexes with receptors and these complexes are biologically active in triggering a persistent increase in membrane-bound adenylate cyclase activity and in increasing bladder permeability to water irreversibly.[4-8] With tritium-labeled derivatives of these analogues it has been possible to characterize the vasopressin receptor from bovine kidney.[9]

Vasopressin analogues are not ideal for studying vasotocin receptors, because of their lower potency in nonmammalian systems. We have, therefore, synthesized photoreactive vasotocin analogues with *p*-azido groups in positions 7[10] and 8.[11] Although both compounds bind covalently to toad bladder receptors and are biologically active, only the vasotocin analogue with the photoreactive group in position 7 is slightly more potent than our best vasopressin photoaffinity analogue.[8]

In the present study we have investigated the possibility of adding photosensitive groups in position 9 of vasotocin. Other studies have already indicated that changes in the C-terminal portion of the acyclic part of vasopressin do not appreciably alter the affinity of iodinated antagonists of vasopressin for V-2 receptors.[12] We have, therefore, synthesized an analogue of vasotocin with *p*-aminophenylalanine in position 9, i.e., 1-desamino[9-(*p*-aminophenylalanine)]AVT, which is a suitable precursor for the attachment of a photoreactive *p*-azido group and can also be used for attachment of a biotinyl residue. Both compounds were found to be considerably more potent in the toad bladder than the corresponding analogues modified in position 7. The photoaffinity analogue—when radiolabeled—is suitable for isolating and characterizing vasotocin receptors, whereas the biotinyl analogue—when bound to an avidin affinity column—can be used to enrich vasotocin receptors prior to photolabeling.

Vasopressin receptor research would be facilitated by the availability of a biologically active radioiodinated vasopressin analogue. It has been shown that the bulky iodine residue on tyrosine in position 2 of the nonapeptide substantially reduces biological activity of the hormone.[12] In the present study we have synthesized an analogue of vasotocin with a tyrosine moiety in position 9 to which iodine was attached. Tyrosine in position 2 was replaced by phenylalanine, i.e., [2-phenylalanine,9-(iodotyrosine)]AVT, to avoid simultaneous iodination at that locus. This compound was found to be biologically active at a concentration of 10^{-9} M, so that this analogue can serve as a probe for vasotocin receptors when radiolabeled.

The analogues of vasotocin that we have synthesized have the general structure shown in Figure 1.

Results

Chemistry. The three analogues **1–3** (Figure 1) were synthesized by the solid-phase method[14,15] on 1% ben-

* Address correspondence to this author at the Department of Physiology and Biophysics, Mount Sinai Medical School, 1 Gustave Levy Place, New York, NY 10029.

† Abbreviations follow the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Int. J. Pept. Protein Res.* **1984**, *24*, 19). Additional abbreviations: TFA, trifluoroacetic acid; DMF, dimethylformamide; DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Me(Bzl), methylbenzyl; Z, benzyloxycarbonyl; β-Mpa, β-mercaptopropionic acid; Phe(pNH$_2$), *p*-amino-L-phenylalanine; d, 1-desamino; HPLC, high-performance liquid chromatography.

(1) Sawyer, W. H. *Am. J. Med.* **1967**, *42*, 678.
(2) Acher, R. *Handb. Physiol.* **1974**, *4*, 119.
(3) Bentley, P. *J. Endocrinol.* **1958**, *17*, 201.
(4) Eggena, P.; Fahrenholz, F.; Schwartz, I. L. *Endocrinology* **1983**, *113*, 1413.
(5) Fahrenholz, F.; Gabor, T.; Crause, P.; Eggena, P.; Schwartz, I. L. *J. Biol. Chem.* **1983**, *258*, 14861.
(6) Eggena, P.; Ma, C. L.; Fahrenholz, F.; Schwartz, I. L. *Am. J. Physiol.* **1985**, *249*, C84.
(7) Buku, A.; Schwartz, I. L.; Gazis, D.; Ma, C. L.; Eggena, P. *Endocrinology* **1985**, *117*, 196.
(8) Eggena, P.; Ma, C. L.; Fahrenholz, F.; Schwartz, I. L. *Am. J. Physiol.* **1986**, *251*, C443.
(9) Fahrenholz, F.; Boer, R.; Crause, P.; Toth, M. V. *Eur. J. Biochem.* **1985**, *252*, 589.
(10) Eggena, P.; Buku, A.; Ma, C. L.; Wyssbrod, H. R.; Gazis, D.; Glass, J. D. *Endocrinology* **1987**, *121*, 2245.
(11) Fahrenholz, F.; Eggena, P.; Kojro, E.; Gazis, D.; Schwartz, I. L. *Int. J. Pept. Protein Res.* **1987**, *30*, 577.
(12) Jard, S.; Lombard, C.; Sawyer, W. H.; Aumelas, A.; Manning, M.; Sawyer, W. H. *Mol. Pharmacol.* **1987**, *32*, 369.
(13) Manning, M.; Coy, E. J.; Acosta, M.; Sawyer, W. H. *J. Med. Chem.* **1973**, *16*, 463.
(14) Merrifield, R. B. *Biochemistry* **1964**, *3*, 1385.
(15) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

0022-2623/89/1832-2432$01.50/0

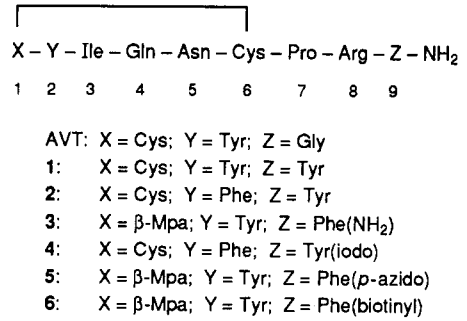

Figure 1. Structures of vasotocin analogues synthesized.

Table I. Hydroosmotic Toad Bladder Activity of Vasotocin Analogues[a]

compound	concn for half-maximal response (ED-50), M
[8-arginine]vasotocin	$(2.2 \pm 0.9) \times 10^{-11}$ ($n = 4$)
1-desamino[9-(*p*-aminophenylalanine)]-vasotocin (**3**)	$(1.3 \pm 0.4) \times 10^{-10}$ ($n = 16$)
1-desamino[9-(*p*-azidophenylalanine)]-vasotocin (**5**)	$(0.9 \pm 0.6) \times 10^{-10}$ ($n = 6$)
1-desamino[9-(biotinylphenylalanine)]-vasotocin (**6**)	$(1.2 \pm 0.4) \times 10^{-8}$ ($n = 8$)
[9-tyrosine]vasotocin (**1**)	$(4.9 \pm 3.3) \times 10^{-10}$ ($n = 3$)
[2-phenylalanine,9-tyrosine]vasotocin (**2**)	$(2.1 \pm 0.9) \times 10^{-9}$ ($n = 6$)
[2-phenylalanine,9-(iodotyrosine)]-vasotocin (**4**)	$(2.5 \pm 1.4) \times 10^{-9}$ ($n = 14$)

[a] Values are the means and standard errors of the mean on the number of toads given in parentheses.

zhydrylamine resin[16] by using the N^{α}-Boc and benzyl side-chain protection for cysteine, mercaptopropionic acid, and tyrosine. Tosyl was used for side-chain protection for arginine. All couplings were in dimethylformamide with dicyclohexylcarbodiimide/1-hydroxybenzotriazole as the coupling reagent.[17] Peptides were cleaved from the resin with hydrogen fluoride/anisole, 9:1, and the sulfhydryl groups oxidized with diiodoethane[18] to form the cyclic disulfides. They were purified by gel filtration and checked for homogeneity by thin-layer chromatography, reverse-phase HPLC, and amino acid analysis. The iodo analogue **4** was prepared by iodination of analogue **2** with iodine monochloride.[19] The azido analogue **5** and biotinyl analogue **6** were prepared from the parent compound **3** by diazotization and reaction with sodium azide[20] and by reaction with biotinyl succinimide ester, respectively.[21]

Biological Activity. The biological activities of the vasotocin analogues were tested in the hydroosmotic toad bladder assay as described previously.[22] The concentrations for half-maximal responses (ED-50 values) are shown in Table I for compounds **1–6**. The rat antidiuretic and rat pressor activities for these analogues are shown in Table II.

The photosensitive vasotocin analogue (compound **5**) was irradiated with long-wavelength UV light as detailed previously.[4] As is shown in Figure 2, bladders exposed to the photoaffinity analogue in the presence of UV light show a persistent increase in permeability to water, whereas matched bladders exposed to the analogue in the absence of UV light show a reversible increase in bladder permeability to water. The persistent increase in membrane permeability to water following UV irradiation is not due to the irradiation per se, because bladders irradiated in the presence of vasotocin exhibit a reversible increase in membrane permeability to water (data not shown). Bladders exposed to the biotinyl analogue (compound **6**) became more permeable to water (Figure 3), and this response was rapidly reversed when avidin was added.

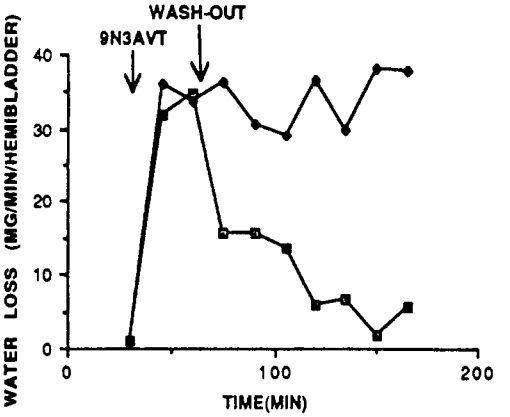

Figure 2. Matched hemibladders were filled with half-strength Ringer's fluid and suspended into full-strength Ringer's fluid containing compound **5** (9N3AVT) at a final concentration of 1×10^{-7} M at $t = 30$ min. One set of hemibladders was irradiated with UV light during a 30-min exposure to analogue, and the other set of hemibladders was not irradiated. Both sets of hemibladders were then removed to a large bath of Ringer's fluid to wash out unreacted analogue. Values given are the mean water fluxes in four experiments. (⊡) No UV irradiation; (♦) with UV irradiation.

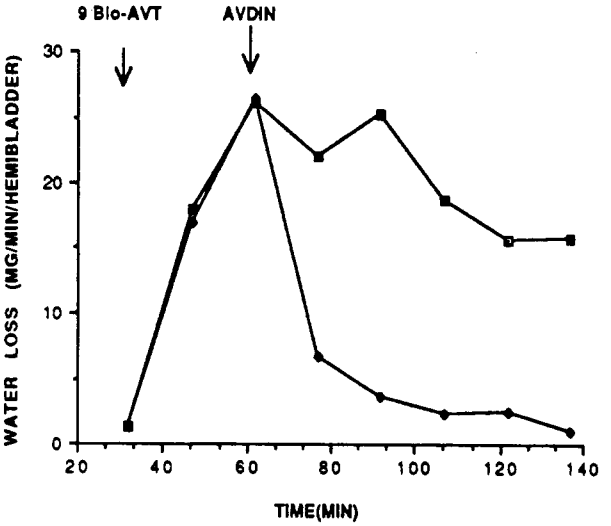

Figure 3. Matched hemibladders were filled with half-strength Ringer's fluid and suspended in full-strength Ringer's fluid containing 1-desamino[9-(biotinylphenylalanine)]AVT (compound **6**, 9Bio-AVT) at a final concentration of 7×10^{-8} M. After 30 min, avidin (0.1 units/mL) was added to the outside bathing solution of one set of hemibladders in the continued presence of the biotinyl analogue. Values are the mean water fluxes of four experiments. (⊡) No avidin; (♦) with avidin.

Discussion

The present studies shows that analogues of vasotocin with major modifications in position 9 are biologically active in increasing toad bladder permeability to water.

(16) Pietta, P. G.; Cavallo, P. F.; Takahashi, K.; Marchall, G. R. *J. Org. Chem.* **1974**, *39*, 44.
(17) Konig, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788.
(18) Weygand, F.; Zumach, G. *Z. Naturforsch, B* **1962**, *B167*, 807.
(19) Fahrenholz, F.; Kojro, E.; Plage, G.; Mueller, M. *J. Recept. Res.* **1984**, *8*, 283.
(20) Fahrenholz, F.; Thierauch, K. H.; Crause, P. *Hoppe-Seyler's Z. Physiol. Chem.* **1980**, *361*, 153.
(21) Buku, A.; Eggena, P.; Wyssbrod, H. R.; Schwartz, I. L.; Gazis, D.; Somoza, L.; Glass, J. D. *J. Med. Chem.* **1987**, *30*, 1526.
(22) Eggena, P.; Schwartz, I. L.; Walter, R. *J. Gen. Physiol.* **1970**, *56*, 250.

Table II. Rat Antidiuretic and Pressor Activities of Vasotocin Analogues

compound	antidiuretic[a]	pressor[a]
[8-arginine]vasotocin	231 ± 30[b]	160 ± 4[b]
[9-tyrosine]vasotocin (1)	25 ± 3.6	0
[2-phenylalanine,9-tyrosine]vasotocin (2)	2.8 ± 0.6	0
1-desamino[9-(*p*-aminophenylalanine)]vasotocin (3)	66 ± 13	0
[2-phenylalanine,9-(iodotyrosine)]vasotocin (4)	0.41 ± 0.14	
1-desamino[9-(*p*-azidophenylalanine)]vasotocin (5)	40 ± 7	
1-desamino[9-(biotinylphenylalanine)]vasotocin (6)	23 ± 10	

[a] Units/mg; mean ± SD. [b] Reference 13.

We have previously studied a series of photoreactive vasopressin and vasotocin analogues in the toad bladder.[4-8,10,11] The most potent of these, 1-desamino[7-(azidolysine)]AVT,[10] yielded a half-maximal response at a concentration of 2.4×10^{-8} M. By comparison, 1-desamino[9-(azidophenylalanine)]AVT, synthesized in the present study, yielded a half-maximal response at 9×10^{-11} M, i.e., 100-fold more potent than the best available photoanalogue.

The present results suggest that the high potency of analogues 1–3 can be attributed to the replacement of glycine in position 9 by aromatic amino acids. Structure–activity and solution conformation studies of neurohypophyseal peptide hormones[23] suggest that the three-residue "tail" part of the molecule is important for specificity as well as activity. The aromatic residue, presumably, orients the acyclic part of the molecule in a favorable position relative to the ring portion of the molecule due to hydrophobic interactions. Even after introduction of photoaffinity or iodo residues considerable activity is preserved. On the other hand, addition of the biotinyl residue resulted in a 100-fold decrease in activity. This may be due to the greater bulk of the biotinyl group and/or because of the long spacer arm by which this group is attached to position 9.

The vasotocin analogue iodinated at position 9 (compound 4) was biologically active at low concentrations, i.e., ED-50 value of 2.5×10^{-9} M. With introduction of radiolabeled iodine this would yield an analogue with a high specific radioactivity for receptor isolation studies. Compound 4 can also be converted to a tritiated vasotocin analogue[19] that could be used for vasotocin receptor binding studies.

Another aspect of receptor research involves receptor isolation and receptor visualization and tracking in target tissues. For such purposes the biotinyl analogue (compound 6) can be linked to an avidin column for affinity chromatography and ferritin- and fluorescein-containing avidin can be used to localize receptors in target cells.[26]

Although potent, iodinated antagonists are available for mammalian vasopressin receptor studies, it has not been possible to synthesize equally potent iodinated agonists.[12] The iodinated vasotocin analogue described in the present study also lacks agonistic activity in the rat antidiuretic assay (compound 4, Table II). These observations emphasize the structural difference between the amphibian hydroosmotic receptor and the mammalian antidiuretic (V-2) receptor. Nevertheless, the photoreactive analogue (compound 5) and the biotinyl analogue (compound 6) do have substantial antidiuretic activity and may, therefore, be useful in the isolation and characterization of the mammalian V-2 receptor.

In conclusion, this study describes the synthesis of potent analogues of vasotocin that are suitable for isolation and characterization of vasotocin receptors in toad bladder and in tissues from other species which use this hormone as an antidiuretic/pressor principle. Preliminary results in the rat suggest that some of these analogues may also be suitable probes for mammalian V-2 receptors.

Experimental Section

Thin-layer chromatography (TLC) (loads 10–50 μg; chromatogram lengths 10–130 mm) was performed on silica gel GF (Analtech, Newark, DE). Spots were detected by chlorination followed by treatment with 1% aqueous KI–starch solution. The following solvent systems (v/v) were used: (A) 1-BuOH–pyridine–AcOH–water (15:10:3:6); (B) 1-BuOH–pyridine–AcOH–water (15:10:3:12); (C) 1-BuOH–AcOH–water (4:1:1).

For amino acid analysis the analogues were hydrolyzed for 22 h at 110 °C with 6 N HCl containing 0.3% phenol in sealed, evacuated tubes and were then analyzed on a Beckman 6300 high-performance amino acid analyzer.

Reverse-phase isocratic HPLC analysis was on a Vydac C-18 RP column for peptides and proteins (1 cm × 25 cm) in an Altex–Beckman instrument (322MPLC) connected with a Hitachi 100-400 multiple-wavelength UV detector and a Hewlett-Packard Model 3380A integrator. The solvent mixtures were 10–20% 2-propanol in 0.1% aqueous TFA with a flow rate of 2 mL/min at A_{215} 0.1 AUFS.

Peptides were synthesized by stepwise solid-phase Merrifield techniques. The N^{α}- and side-protected amino acids used, except glycine, were all in the L configuration and were purchased from Bachem (Torrance, CA) or from Peninsula Laboratories (San Carlos, CA). All solvents and reagents were of analytical grade or were purified according to procedures recommended for solid-phase peptide synthesis.[27]

The side-chain protections were N^{G}-tosyl (arginine), 2-Br-Z (tyrosine), Z (*p*-aminophenylalanine), and *S*-(4-methylbenzyl) (L-cysteine and β-mercaptopropionic acid).

Cys(MeBzl)-Tyr-Ile-Gln-Asn-Cys(MeBzl)-Pro-Arg(tosyl)-Tyr(2-Br-Z)-resin, Cys(MeBzl)-Phe-Ile-Gln-Asn-Cys(MeBzl)-Pro-Arg(tosyl)-Tyr(2-Br-Z)-resin, and β-Mercaptopropionyl(MeBzl)-Tyr(2-Br-Z)-Ile-Gln-Asn-Cys(MeBzl)-Pro-Arg(tosyl)-Phe(p-NH-Z)-resin. Syntheses were performed on a Vega 96 automatic peptide synthesizer using BHA resin prepared from polystyrene 1% divinylbenzene (Beckman Chemicals). A well-established synthetic protocol for vasopressin analogues has been utilized in the present work.[28] Briefly, 1 g of 1% cross-linked BHA resin (0.51 mequiv of amine content/g of resin) was subjected to the following deprotection, neutralization, coupling steps, and washings for every synthetic cycle for each amino acid: (step 1) deprotection with 50% TFA–CH_2Cl_2–2% anisole, 1 × 2 min, and 1 × 15 min, followed by washing with CH_2Cl_2 several times; (step 2) neutralization with 0.5% DIEA in CH_2Cl_2, 3 × 2 min, followed by several washings with CH_2Cl_2/i-PrOH and DMF; (step 3) coupling and incorporation of the C-terminal tyrosine or *p*-aminophenylalanine to the resin, 4-fold molar excess of amino acid and HOBt in DMF and

(23) Walter, R.; Smith, C.; Mehta, P.; Boonjarern, S.; Arruda, J.; Kurtzman, N. In *Disturbances in Body Fluid Osmolality*; Andreoli, T., Grantham, J., Rector, F., Jr., Eds.; American *Physiological Society*: Bethesda, MD, 1977; p 1.
(24) Sawyer, W. H. *Endocrinology* **1958**, *63*, 694.
(25) Dekanski, J. *Br. J. Pharmacol.* **1952**, *7*, 567.
(26) Beyer, E. A.; Wilchek, M. *Methods Biochem. Anal.* **1980**, *26*, 1.
(27) Andreu, D.; Merrifield, R. B.; Steiner, H.; Boman, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6475.
(28) Buku, A.; Gazis, D.; Schwartz, I. L. *Int. J. Pept. Protein Res.* **1984**, *23*, 551.

DCC in CH_2Cl_2 preactivated for 10 min at 0 °C, followed by several washings with DMF, i-PrOH, and CH_2Cl_2. The completeness of the reaction was checked by the ninhydrin test,[29] and repeated couplings were performed as indicated.

[Tyr^9]AVT (1), [Phe^2,Tyr^9]AVT (2), and d[*p*-amino-Phe^9]AVT (3). The dried polymer carrier (1.2 g) was cleaved from the peptide with hydrogen fluoride–*p*-cresol (9:1) for 1 h at 0 °C and washed with ether, and the free peptides were extracted from the resin with 0.2 N acetic acid. The acetic acid solution was diluted with degassed methanol–water (2:1) to a volume of 600 mL and adjusted to pH 8 with ammonia, and the sulfydryl bonds were oxidized with diiodoethane in methanol [1 equiv on the basis of the amine content/g of starting resin] to form the cyclic disulfide. The oxidation was monitored by the Ellman Test,[30] and after 10 min, the solution was acidified and reduced in vacuo to a small volume.

The peptides [Tyr^9]AVT and [Phe^2,Tyr^9]AVT were purified by gel chromatography on Sephadex G-15 twice in 50% acetic acid and 0.2 M acetic acid. The d[*p*-amino-Phe^9]AVT was chromatographed twice on Sephadex G-15 with 0.2 M acetic acid and LH 20 with methanol. The following analytical data were obtained.

[Tyr^9]AVT (1): TLC R_f 0.75 (A), 0.64 (B), 0.21 (C); amino acid anal. Asp 0.98; Glu 1.08; Ile 0.97, Cys 1.88, Tyr 1.98, Arg 1.02, Pro 1.09; HPLC t (min) 13.1, k' = 1.41 (10% i-PrOH), single peak, 98% purity.

[Phe^2,Tyr^9]AVT (2): TLC R_f 0.76 (A), 0.64 (B), 0.21 (C); amino acid anal. Asp 1.0, Glu 0.98, Ile 1.06, Cys 1.89, Tyr 0.98, Arg 1.08, Pro 0.98, Phe 0.99; HPLC t (min) 28.99, k' = 3.37 (10% i-PrOH), single peak, 98% purity.

d[*p*-amino-Phe^9]AVT (3): TLC 0.59 (A), 0.73 (B), 0.15 (C); amino acid anal. Asp 1.0, Glu 0.99, Ile 0.99, Cys 0.84, Tyr 0.93, Arg 0.99, Pro 1.09, *p*-amino-Phe 0.99; HPLC t (min) 12.25, k' = 0.83 (10% i-PrOH), single peak, 97.5% purity.

[Phe^2,iodo-Tyr^9]AVT (4). To a solution of 20 mg (2) in 5 mL of 0.1 M ammonium acetate buffer (pH 5.7) was added, while stirring at 0 °C, 0.2 mL of iodine monochloride (1.4 M) in dioxane. After 1 min the reaction was stopped with 0.1 M aqueous hydrazine hydrate and the solution was chromatographed on Sephadex G-15 with 0.2 M acetic acid: yield 7 mg; TLC R_f 0.82 (A), 0.71 (B), 0.43 (C); HPLC t (min) 17.62, k' = 1.64 (20% i-PrOH). The analysis showed that the main product (98%) was the monoiodinated analogue.

d[*p*-azido-Phe^9]AVT (5). Compound 3 (20 mg) was dissolved in 1 mL of 0.1 N HCl and treated with sodium nitrite (0.1 mL of 1 N aqueous solution) for 15 min at 0 °C at pH below 2. Aqueous sodium azide (0.1 mL) was then added and the solution stirred for 30 min at 0 °C. The mixture was then chromatographed on Sephadex G-15 with 0.2 M acetic acid: yield 15 mg; TLC R_f 0.82 (A), 0.75 (B), 0.50 (C); HPLC t (min) 21.87, k' = 2.25 (20% i-PrOH), single peak, 99% purity.

d[biotinyl-Phe^9]AVT (6). Compound 3 (20 mg) was dissolved in 2 mL of DMF and treated at pH 7.5 (*N*-methylmorpholine as base) with 15 mg of biotinyl succinimide ester (Pierce) overnight at room temperature. The solution was evaporated in vacuo and the residue chromatographed on Sephadex G-15 with 0.2 M acetic acid: yield 10 mg; TLC R_f 0.83 (A), 0.82 (B), 0.45 (C); HPLC t (min) 12.1, k' = 0.75 (10% i-PrOH), single peak, 98% purity.

Hydroosmotic Toad Bladder Assay. Toads (*Bufo marinus*; Dominican Republic), weighing 150–250 g, were doubly pithed, and their urinary bladders were resected and tied to the ends of glass cannulae with the mucosa facing inward. Hemibladders were suspended in Ringer's fluid of the following composition, in mmol/L: NaCl, 110; KCl, 3.5; $CaCl_2$, 1; $MgCl_2$, 1; dextrose, 5.5; and tris(hydroxymethyl)aminomethane hydrochloride, 10. The pH of this solution was 7.5 and its osmolality was 235 mosmol/kg of water, as determined with a Wescor vapor pressure osmometer (Wescor Inc., Logan, UT). Hemibladders were filled with Ringer's fluid diluted to half-strength with distilled water. Net osmotic water flux across the bladder wall was measured gravimetrically according to the method of Bentley.[3] The protocol used for determining the effective dose for a half-maximal water flow response in Table I has been detailed previously.[22]

The procedure used for photolabeling toad bladders was as described previously.[4] Hemibladders were placed—four at a time—into 100 mL of Ringer's fluid containing the photoaffinity analogue, and the baths were irradiated with long-wavelength UV light (365 nm) from above and below using a Blak-Ray lamp (Model XX-15, Ultraviolet Products, Inc., San Gabriel, CA). The intensity of UV irradiation was monitored with a UV light meter to assure equivalent conditions of irradiation for control and experimental hemibladders. Irradiation was carried out for 30 min. Following irradiation hemibladders were removed to large 300-mL baths of Ringer's fluid to wash out any unreacted analogue. Both the outside and inside bathing solutions were changed at 30-min intervals.

Rat Antidiuretic Assay. Antidiuretic activities were assayed on water-loaded rats, according to the method of Sawyer.[24]

Rat Pressor Assay. Pressor activities were assayed on urethane-anesthetized, phenoxybenzamine-treated rats, as described by Dekanski.[25]

Acknowledgment. We thank Ms. Janice Walcott and Mr. Robin Gehrman for technical assistance with this study. This work was supported by Grant DK35356 from the National Institute of Diabetes and Digestive and Kidney Diseases (P.E.) and Grant HD19517 from the National Institute of Child Health and Human Development (D.G.).

Registry No. 1, 121920-73-8; 2, 121935-31-7; 3, 121920-74-9; 4, 121935-32-8; 5, 121920-75-0; 6, 121920-76-1; *d*-biotinyl succinimide ester, 35013-72-0.

(29) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
(30) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.