Design, Synthesis, and Biological Activity of a Peptide Mimic of Vasopressin[1]

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Our molecular modeling studies suggested that the conformational effects of the "cystine-line" residue Pmp[1]-Cys[6] on the cyclohexapeptide ring of the vasopressin antagonist [Pmp[1],D-Phe[2],Val[4]]AVP might be mimicked by substitution of D-aminoadipic acid at position 6 and cyclization of its side-chain carboxyl to the α-amine of residue 2. The peptide was prepared with DL-aminoadipic acid, and following cyclization, the two diastereomeric peptides were separated and purified by preparative high-performance liquid chromatography. The structure of each was confirmed by amino acid analysis and fast atom bombardment mass spectrometry. The chirality of the aminoadipic acid residue of each peptide was determined by chiral gas chromatography. The circular dichroism spectrum of each peptide was run and compared with the appropriate agonist and antagonist peptide standards. These peptides demonstrated in vitro poor V_2-receptor affinity and an inability to inhibit or stimulate vasopressin-induced adenylate cyclase formation, suggesting that they lack one or more key features of the agonist/antagonist pharmacophore.

We have been interested in determining the biologically active conformation of vasopressin agonists and antagonists and in defining the structural requirements necessary for antagonist activity. To reduce the complexity of searching the conformational space available to a molecule the size of vasopressin, we have attempted to determine a minimum active fragment. As well as helping to define the pharmacophore, this fragment could be used as a conformational model to aid in the design of more specific and potent antagonists. This approach has been used successfully with somatostatin[2] and enkephalin.[3] It is hoped that such studies may lead to the development of peptide or nonpeptide analogues with increased potency or oral activity.

The arginine-vasopressin (AVP) antagonist investigated, **1**, has β-mercapto-β,β-pentamethylenepropionic acid (Pmp) substituted for Cys[1], D-Phe substituted for Tyr[2], and Val substituted for Gln[4]. The activity of this analogue and related vasopressin antagonists was first reported by Manning and co-workers.[4,5] We now report molecular modeling studies that lead to the synthesis of a reduced ring mimic of **1** and a comparison of its in vitro biological activity to **1** as well as to the agonist, **2**, containing β-mercaptopropionic acid (Mpr) at position 1.

X-Y-Phe-Z-Asn-Cys-Pro-Arg-Gly-NH_2

AVP: X = Cys, Y = Tyr, Z = Gln

(Pmp[1],D-Phe[2],Val[4])AVP (**1**): X = Pmp, Y = D-Phe, Z = Val

(Mpr[1],D-Phe[2],Val[4])AVP (**2**): X = Mpr, Y = D-Phe, Z = Val

Molecular Modeling

In several ring conformations of vasopressin derived from molecular dynamics[6] we observed a close proximity between the α-carbons of the cystine residue (Cys[1]-Cys[6]) which suggested that this distance could be spanned by two methylene units without greatly perturbing the remaining ring conformation. A similar substitution in the antagonist, **1**, would result in a reduced ring mimic in which the β-carbons of Pmp[1] and Cys[6] and all their appendages had been removed. Starting with one minimum energy conformer of vasopressin, we constructed[7] the ring conformation of **1** shown in Figure 1. We built in a D-aminoadipic acid (Aad) residue at position 6 with its side-chain carboxyl attached to the amine of the D-Phe at position 2. The resulting structure, **3**, was minimized and

D-Phe-Phe-Val-Asn-D-Aad-Pro-Arg-Gly-NH_2

[desCys[1],cyclo(D-Phe[2],D-Aad[6]),Val[4]]AVP (**3**)

compared to the ring conformation of **1**. As shown in Figure 1, the antagonist **1** may assume ring conformations in which the α-hydrogen of Cys[6] is oriented toward the "interior" of the hexapeptide ring. Because of the D configuration of the Aad, **3** can adopt ring conformations in which the Aad side chain can also point toward the "interior" of the ring. This suggested that, although neither conformation represents a unique low-energy conformation, **3** might be able to exhibit a class of ring conformations available also to **1**.

Chemistry

All peptide analogues were prepared by solid-phase synthesis[8] on benzhydrylamine resin prepared by an improved procedure.[9] The *tert*-butyloxycarbonyl (Boc) group was used for α-amine protection. Amino acid side chains were protected with 4-methylbenzyl for sulfhydryl, tosyl for guanadinyl, and benzyl for carboxyl. The 4-methylbenzyl-protected mercaptopropionic acid (Mpr) was prepared from bromopropionic acid and 4-methylbenzyl mercaptan. The δ-benzyl ester of Aad was prepared as previously described[10] and the Boc group introduced with

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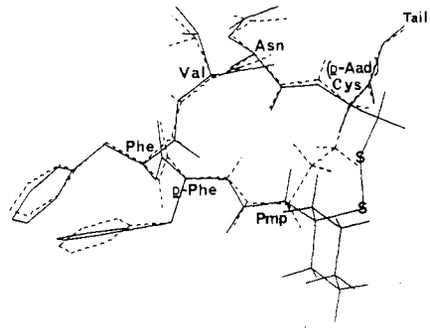

Figure 1. Energy-minimized ring conformation of **1** (–) and **3** (- -).

Table I. In Vitro Biological Activity of Peptides 1–4

no.	compound[a]	K_{bind},[b] M	K_i,[c] M
1	[Pmp^1,D-Phe^2,Val^4]AVP	1.3×10^{-7}	4.6×10^{-8}
2	[Mpr^1,D-Phe^2,Val^4]AVP	1.0×10^{-7}	1.0×10^{-8} (K_a)
3	[desCys^1,cyclo(D-Phe^2,D-Aad^6),-Val^4]AVP	2.9×10^{-5}	9.2×10^{-6}
4	[desCys^1,cyclo(D-Phe^2,Aad^6),-Val^4]AVP	1.5×10^{-5}	7.0×10^{-6}

[a] Abbreviations: Pmp, β-mercapto-β,β-pentamethylenepropionic acid; Mpr, β-mercaptopropionic acid; Aad, aminoadipic acid; All amino acids are of the L configuration unless otherwise noted. [b] Binding affinity to a pig renal medullary preparation as measured by competition with tritiated LVP. [c] Inhibition (K_i) or activation (K_a) of LVP-sensitive adenylate cyclase in a pig renal medullary preparation.

di-*tert*-butyl dicarbonate. All couplings were performed with 1,3-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt). Protected peptide resins were cleaved with hydrogen fluoride (HF). Peptide **3** was prepared with Boc-DL-Aad(Bzl), and following HF cleavage, the linear peptides were cyclized with diphenyl phosphorazidate. The two resulting diastereomeric peptides, **3** and **4**, were separated and purified by high-performance liquid chromatography (HPLC), and the chirality of the Aad residue in each was determined by chiral gas chromatography (GC) of the peptide hydrolyzate. Following HF cleavage from the resin, peptide **2** was oxidatively cyclized with potassium ferricyanide and purified by countercurrent distribution.

Bioassay Methods

Vasopressin V_2 receptor binding was measured by competition with tritiated lysine vasopressin (LVP) in a pig renal medullary preparation and is expressed as a binding constant (K_{bind}).[11] Inhibition of LVP-sensitive adenylate cyclase, as measured in the same preparation, is expressed as an inhibition constant (K_i). Agonist activity, also determined in the same preparation, represents the activation of LVP-sensitive adenylate cyclase and is expressed as an activation constant (K_a).[11]

Results and Discussion

The biological activities of the peptides are given in Table I. As can be seen, peptides **3** and **4** are weak antagonists with poor affinity for the vasopressin V_2 receptor. Their lack of V_2-receptor affinity and inability to inhibit or stimulate vasopressin-induced adenylate cyclase formation suggest that they do not present one or more key elements of the agonist/antagonist pharmacophore. The CD spectra (Figure 2) of peptides **3** and **4** are nearly identical. The spectra show that, when compared to peptide **1**, the changes effected in peptides **3** and **4** have resulted in alterations to the overall peptide solution conformation. In particular, the pronounced negative ellipticity observed at 238 nm in the antagonist, **1**, has been greatly attenuated. The agonist, **2**, shows no negative Cotton effect at 238 nm and in general its spectrum spectrum of AVP.[12,13] The poor receptor affinity indicates that either the loss of the Pmp ring and its disulfide to Cys^6 or the conformational change this deletion has caused have resulted in the loss of one or more important binding elements. The CD and biological data imply that the conformational constraints imposed on the cyclohexapeptide ring by the bulk of the Pmp residue, which may be responsible for turning agonist **2** into antagonist **1**, cannot be mimicked by amide bond formation between the Aad and the D-Phe in peptides **3** and **4**. Alternatively, the Pmp and its disulfide to Cys^6 may themselves represent key features of the agonist/antagonist pharmacophore. These results may indicate that the ring conformation of peptide **1** we attempted to mimic may not be appropriate or peptide **3** may not correctly mimic the conformation of peptide **1** we modeled.

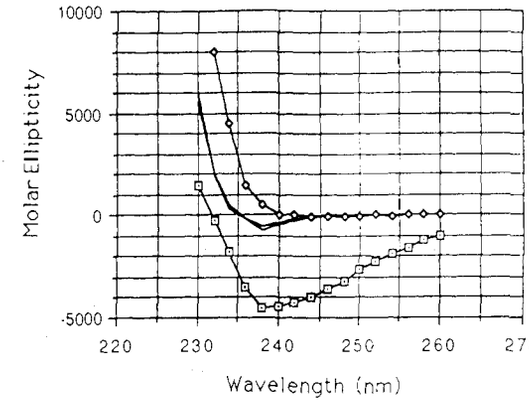

Figure 2. Partial CD spectra of the antagonist **1** (□), peptide **3** (–), peptide **4** (–), and the agonist **2** (◇).

Experimental Section

Protected amino acids were purchased from Peninsula Laboratories or Chemalog, Inc. Aminoadipic acid (Aad) was purchased from Sigma Chemical Co. Peptide **1** was purchased from Peninsula Laboratories. Benzhydrylamine resin (BHA resin, 1% cross-linked S-DVB, 200–400 mesh, ~1.0 mequiv of N/g of resin) was prepared according to Bryan.[9] Solvents and reagents were reagent grade. Methylene chloride (CH_2Cl_2) was HPLC grade and dimethylformamide (DMF) was stored over 4-Å molecular sieves and filtered prior to its use. The purity of all peptides was routinely checked by high-performance liquid chromatography (HPLC) using a 4.5 mm × 25 cm Altex Ultrasphere 5 μ ODS column with UV detection at 220 nm and thin-layer chromatography (TLC) on EM silica gel plates with visualizaton with 10% Clorox–1% KI–starch. For preparative HPLC, a 10 mm × 25 cm column was employed. For analytical HPLC, a linear gradient of water–acetonitrile containing 0.1% trifluoroacetic acid (TFA) was used (80:20 to 50:50 over 30 min at 1.5 mL/min) and preparative HPLC was performed isocratically with 30:70 acetonitrile–water containing 0.1% TFA at 4.0 mL/min. For amino acid analysis peptides were hydrolyzed for 2–3 h at 130 °C in concentrated hydrochloric acid–propionic acid (1:1 v/v).[14] For gas chromatography (GC), peptides were hydrolyzed for 18 h at 110 °C in 6 N hydrochloric acid, and the hydrolyzate was converted to the *N*-pentafluoropropionyl amino acid isopropyl esters

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with 2-propanol–hydrochloric acid followed by pentafluoropropionic acid anhydride according to the procedure of Pisano.[15] Analyses were performed on a Carlo Erba capillary GC with flame-ionization detection and a Chirasil-Val column. The program employed a flow of 19 cm/s at 90 °C for 4 min; 90–170 °C at 4 °/min and 170 °C for 20 min. Amino acid analyses were performed on a Kontron Liquimat III amino acid analyzer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM390 spectrometer using tetramethylsilane as an internal standard. CD spectra were recorded on a JASCO J500-C spectropolarimeter interfaced to an IBM XL computer. Each spectrum was the result of at least eight computer averaged scans with background correction. All peptides were run at a concentration of 0.1 mg/mL in Tris buffer, pH 8.0. Elemental analyses were performed on a Perkin-Elmer 240 apparatus. Where analyses are reported by symbols of elements, results were within 0.4% of the calculated values. Melting points were determined in open capillary tubes with a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Fast atom bombardment (FAB) mass spectrometry was performed by the Analytical, Physical and Structural Chemistry Department of Smith Kline & French Laboratories on a VG ZAB high-resolution spectrometer.

δ-Benzyl *N*-(*tert*-Butyloxycarbonyl)-DL-aminoadipate. The δ-benzyl ester of Aad[10] (1.23 g, 5 mmol) was suspended in 25 mL of DMF and triethylamine was added (0.51 g, 5 mmol). To this solution was added di-*tert*-butyl dicarbonate (1.2 g, 5.5 mmol) and the reaction mixture was stirred at room temperature for 48 h. The solution was concentrated and the residue dissolved in ethyl acetate. The ethyl acetate solution was washed with water, saturated potassium hydrogen sulfate, and brine. It was dried over magnesium sulfate, filtered, and concentrated to yield 0.55 g of an oil: NMR ($CDCl_3$) δ 1.4 (s, $C(CH_3)$), 1.6–1.9 (m, 2 CH_2), 2.3 (t, CH_2CO_2), 3.9 (m, COCHN), 4.1 (br db, NH), 5.1 (s, $ArCH_2$), 7.3 (s, Ar H); FAB mass spectrum (DCHA salt), m/z 533 $(M + H)^+$.

3-[(4-Methylbenzyl)thio]propionic Acid. To a solution of sodium hydroxide (30 g, 0.75 mol) in 300 mL of water was added 4-methylbenzyl mercaptan (28.8 g, 0.209 mol). The mixture was stirred under argon for 1 h before 3-bromopropionic acid (30.6 g, 0.2 mol) was slowly added. When the addition was complete, the reaction mixture was stirred at 45 °C for 4 h and then at room temperature for 12 h. The reaction mixture was diluted with 300 mL of water and washed with ether–hexane (1:1 v/v). The aqueous phase was acidified to pH 2 with concentrated phosphoric acid, and the resulting solid collected and washed with cold water. Recrystallization from hexane provided 39.4 g of product: mp 74–75 °C; NMR ($CDCl_3$) δ 2.3 (s, CH_3), 2.6 (m, 2 CH_2), 3.7 (s, $ArCH_2S$), 7.2 (m, Ar H). Anal. ($C_{11}H_{14}O_2S$) C, H.

General Solid-Phase Synthesis of Peptides. Peptides were synthesized on 1.0–2.0 g of BHA resin. All couplings were performed with 3 equiv of the Boc-protected amino acid with 1,3-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) in DMF for 2–12 h. The Boc group was removed with a single 20-min treatment with 50% TFA in CH_2Cl_2. The resulting amine TFA salt was neutralized with two 2-min treatments of 7% diisopropylethylamine (DIEA) in CH_2Cl_2. Protected peptide resins were cleaved and deprotected with anhydrous hydrogen fluoride (HF) with 10% anisole at 0 °C for 1 h. After removal of the HF in vacuo at 0 °C, the residue was washed with diethyl ether and the peptide extracted with acetic acid or acetic acid–water.

[Pmp[1],D-Phe[2],Val[4]]AVP (1): HPLC, $k' = 10.35$; TLC, 1-butanol–ethyl acetate–acetic acid–water, 1:1:1:1 v/v/v/v, R_f 0.57; amino acid analysis, Asp 1.03, Pro 0.95, Gly 1.00, Cys 0.61, Val 1.00, Phe 1.85, Arg 0.93.

[Mpr[1],D-Phe[2],Val[4]]AVP (2). The solution of the crude uncyclized peptide in acetic acid (HOAc) was added to 3 L of water and the pH adjusted to 7.5 with concentrated ammonium hydroxide. With stirring, a 0.01 M solution of potassium ferricyanide was added dropwise until a faint yellow color persisted for 30 min. The solution was then adjusted to pH 4.5 with HOAc and filtered. It was then passed through a BioRex 70 ion exchange column (H^+). The column was washed with water (~300 mL) and then the peptide was eluted with pyridine–acetic acid–water (30:4:66 v/v/v). The eluant was evaporated and the residue lyophilized from 0.2 N HOAc. The crude peptide was purified by countercurrent distribution in 1-butanol–acetic acid–water (4:1:5 v/v/v). Tubes were checked by TLC and the appropriate fractions pooled, evaporated to dryness, and lyophilized from 0.2 N HOAc to yield 87 mg of peptide: HPLC, $k' = 9.76$; TLC, 1-butanol–acetic acid–water, 4:1:1 v/v/v (BAW), R_f 0.43; FAB mass spectrum, m/z 1024 $(M + H)^+$; amino acid analysis, Asp 0.96, Pro 0.92, Gly 1.00, Cys 0.47, Val 1.38, Phe 1.87, Arg 0.95.

[DesCys[1],D-Phe[2],Val[4],DL-Aad[6]]AVP. After lyophilization, the crude peptides were subjected to gel filtration on G-15 column eluting with 0.2 N HOAc to yield 394 mg of peptide: HPLC, $k' = 5.88$ (the D and L isomers were not resolved); TLC, 1-butanol–pyridine–acetic acid–water, 15:10:3:12 v/v/v/v (BPAW), $R_f = 0.63$; FAB mass spectrum, m/z 978 $(M + H)^+$.

[DesCys[1],cyclo(D-Phe[2],DL-Aad[6]),Val[4]]AVP (3, 4). The linear peptides (150 mg, 0.153 mmol) were converted to the hydrochloride salt by concentration twice at room temperature from 0.2 N HOAc–concentrated HCl (9:1 v/v). The residue was diluted with water and lyophilized. The peptides were dissolved in 100 mL of DMF and the pH adjusted to ~7.5 (moistened pH paper) with triethylamine. The solution was cooled to 0 °C and diphenyl phosphorazidate (200 μL, 0.93 mmol) was added in one portion with stirring. The reaction mixture was stirred at 0 °C for 4 h and then overnight at room temperature. The mixture was concentrated at 30 °C, diluted with 0.2 N HOAc, and lyophilized. The peptides were then purified by preparative HPLC to yield 32 mg of the L isomer and 40 mg of the D isomer as determined by chiral GC: TLC (BPAW), R_f 0.69 (the D and L isomers were not resolved); L isomer, analytical HPLC, $k' = 8.98$; FAB mass spectrum, m/z 960 $(M + H)^+$; amino acid analysis, Asp 1.00, Pro 1.02, Gly 1.00, Aad 1.09, Val 0.93, Phe 1.81, Arg 0.96; L-Pfp-Aad-iPr GC retention time of 31.28 min; D isomer, analytical HPLC, $k' = 7.46$; FAB mass spectrum, m/z 960 $(M + H)^+$; amino acid analysis, Asp 1.03, Pro 0.99, Gly 1.00, Aad 1.07, Val 0.88, Phe 1.92, Arg 0.95; D-Pfp-Aad-iPr GC retention time of 31.80 min.

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Registry No. 1, 81094-03-3; 2, 112219-70-2; 2 (uncyclized), 112246-95-4; 3, 112219-71-3; 3 (uncyclized), 112219-73-5; 4, 112219-72-4; 4 (uncyclized), 112219-74-6; δ-benzyl *N*-(*tert*-butyloxycarbonyl)-DL-aminoadipate, 112295-37-1; δ-benzyl DL-aminoadipate, 80446-57-7; 3-[(4-methylbenzyl)thio]propionic acid, 112219-75-7; 4-methylbenzyl mercaptan, 4498-99-1; 3-bromopropionic acid, 590-92-1.

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