Chemical Syntheses and Biological Studies on Dimeric Chimeras of Oxytocin and the V_2-Antagonist, d$(CH_2)_5$[D-Ile[2],Ile[4]]arginine Vasopressin[||,∇]

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Parallel and antiparallel heterodimers have been synthesized that combine into a single molecule the neurohypophyseal hormone oxytocin and the potent vasopressin V_2-antagonist d$(CH_2)_5$[D-Ile[2],Ile[4]]arginine vasopressin. Solid-phase synthesis with N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, featuring appropriate combinations of orthogonal protecting groups for the thiols [*S*-(*N*-methyl-*N*-phenylcarbamoyl)sulfenyl (Snm); *S*-acetamidomethyl (Acm); *S*-triphenylmethyl (Trt)], was used to assemble the required linear nonapeptide amide monomer intermediates, which were then brought together in defined ways by solution reactions to provide the two heterodimers. The first disulfide bridge was formed by a directed approach involving attack by the free thiol of the 1-β-mercapto-β,β-cyclopentamethylenepropionic acid (Pmp) residue of one monomer onto the Snm group of a cysteine residue on the other monomer; the inverse directed strategy failed due to steric hindrance. The second disulfide bridge was formed by iodine co-oxidation of Cys(Acm) residues on adjacent chains. Biological studies revealed that both the parallel and antiparallel chimeras lack pressor activity, have low uterotonic activity, and have diuretic activities comparable to that of the monomeric V_2-antagonist. Sodium excretion depends on experimental conditions. Thus, with a 4% water load, both chimeras display effects similar to that of an equimolar mixture of oxytocin and V_2-antagonist, i.e., lower sodium excretion than that resulting from administration of oxytocin alone but higher than that when V_2-antagonist was administered alone. However, when no water load was used, the parallel chimera proved to be more effective in promoting sodium excretion than either oxytocin alone or an equimolar mixture of oxytocin and V_2-antagonist.

Introduction

Starting with the initial syntheses of oxytocin and vasopressin reported by du Vigneaud and co-workers[1,2] in 1954, these two neurohypophyseal hormones, as well as their agonists and antagonists, have been the subject of extensive pharmacological investigations. Hundreds of analogues of these hormones have been synthesized,[3,4] and a number of them are used worldwide as valuable tools in medicine, pharmacology, and physiology.[5,6] Vasopressin acts primarily as an antidiuretic and pressor agent, whereas oxytocin has potent uterotonic and galactogogic actions. In addition, oxytocin plays a significant physiological role in the regulation of salt and water balance.[7] The optimal doses for the natriuretic effect are between 0.1 and 1 μg of oxytocin/kg of animal weight;[8] at higher doses, detection of this effect is complicated by simultaneous antidiuretic action. Several oxytocin analogues have been designed and synthesized which showed enhanced natriuresis, but all of these were also antidiuretic.[8,9] The recent discovery of oxytocin receptors in the heart[10−12] has led McCann and co-workers to suggest that intracardiac oxytocin binding to its receptors stimulates the release of atrial natriuretic factor (ANF), which mediates the natriuretic effect.

Our laboratories have long been interested in elucidating the significance of disulfide bridges in the actions of peptide hormones.[13−21] A major aspect of our studies has been the directed synthesis and pharmacological evaluation of dimers of neurohypophyseal hormones.[17,19] Dimers of oxytocin were observed originally by Ressler[22] and isolated by Yamashiro, Hope, and du Vigneaud as byproducts from the oxidation step to close the heterodetic ring.[23] Furthermore, Aanning and Yamashiro reported an ingenious intentional synthesis of the parallel dimer.[24] Relatedly, dimers were isolated as

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[∇] Abbreviations used for amino acids and the designation of peptides follow the rules of the IUPAC−IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: Acm, *S*-acetamidomethyl; AVP, arginine vasopressin; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetraethyluronium hexafluorophosphate; HOAc, acetic acid; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; Meb, methylbenzyl; Mpa, β-mercaptopropionic acid; NMM, *N*-methylmorpholine; PAL, “peptide amide linker” [5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid]; PEG-PS, poly(ethylene glycol)−polystyrene (resin support); Pfp, pentafluorophenyl; Pmp, 1-β-mercapto-β,β-cyclopentamethylenepropionic acid; Scm, *S*-methyloxycarbonylsulfenyl; Snm, (*N*-methyl-*N*-phenylcarbamoyl)sulfenyl; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, triphenylmethyl. All amino acids used were of the L-configuration unless indicated otherwise.

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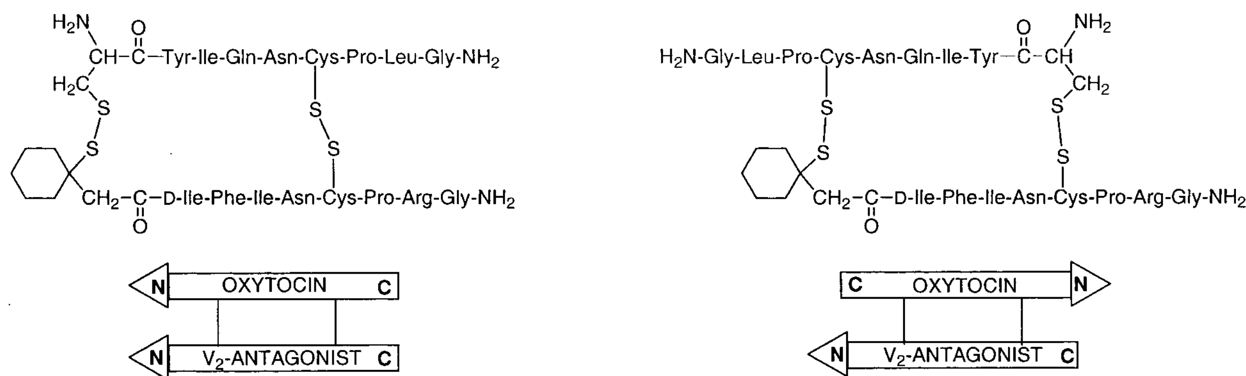
Scheme 1[a]



Parallel heterodimer of oxytocin and the V_2-antagonist. **Antiparallel heterodimer of oxytocin and the V_2-antagonist**

[a] The covalent structures, using standard amino acid abbreviations, are shown on top. A shorthand representation to indicate the orientations of chains, with the arrowheads representing the N-termini, is shown on the bottom.

byproducts during the syntheses of [2-*O*-methyltyrosine]-oxytocin[25] and deamino[8-D-arginine]vasopressin.[26] Also, dimers of lysine vasopressin were isolated (inconclusive as to whether parallel or antiparallel) either from a hypophysial extract or upon repeated lyophilization of the monomer from a slightly alkaline solution, as described by Schally and Guillemin.[27] The isolation of a naturally occurring antiparallel dimeric vasotocin analogue by Proux and colleagues,[28] and confirmation of its structure by unambiguous chemical synthesis of both parallel and antiparallel forms, is of further interest.

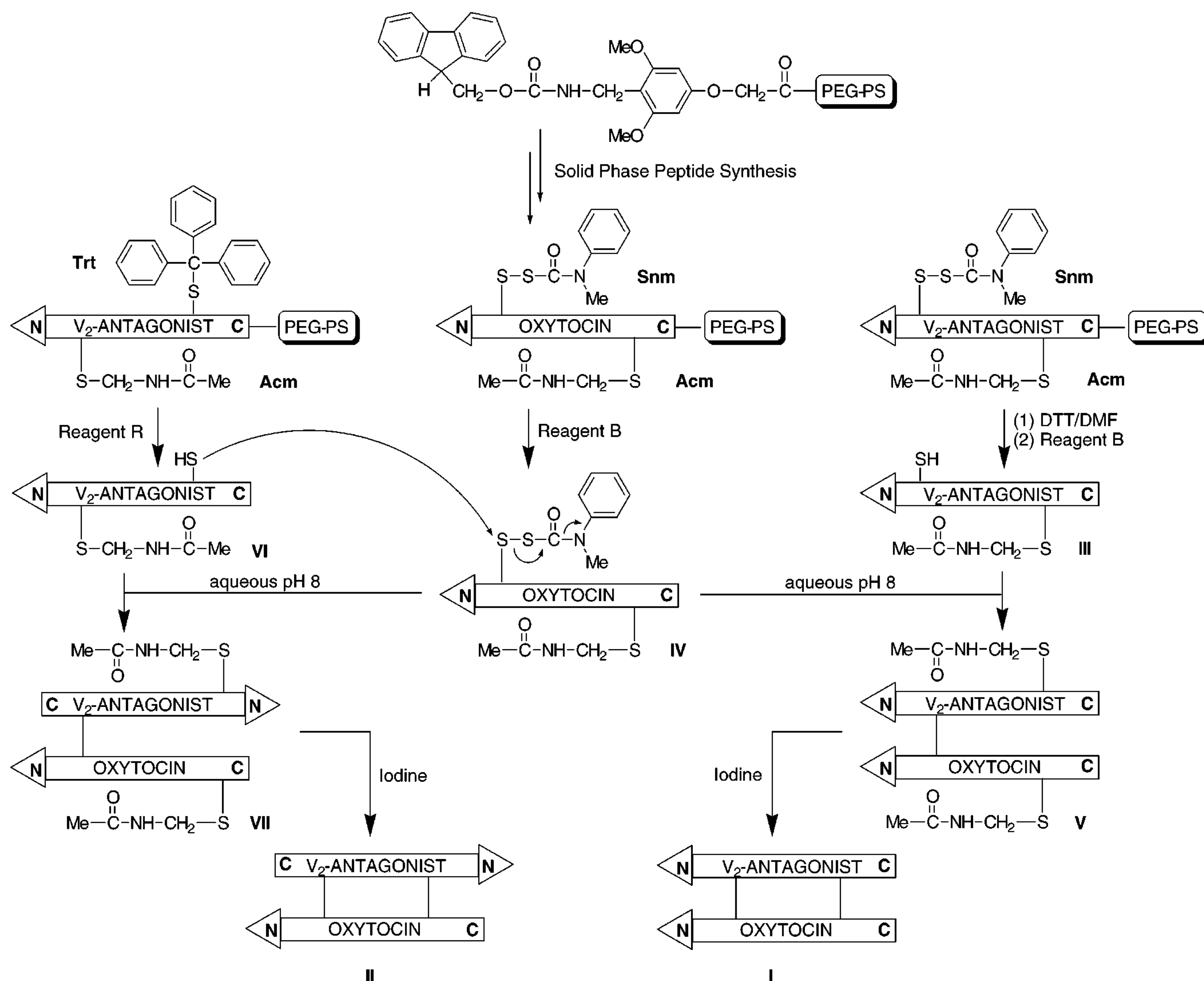
We have described previously efficient methods to synthesize directly parallel and antiparallel homo- and heterodimers of oxytocin and deaminooxytocin.[17,19] These dimers were evaluated in a series of biological assays[17,19] which gave results that were consistent with the hypothesis that dimers exhibit their biological activities after dissociation into monomers. Continuing in this vein, the present paper reports the design and synthesis of both parallel and antiparallel heterodimers (**I** and **II** in Scheme 1) that combine oxytocin and the potent vasopressin V_2-antagonist $d(CH_2)_5$[D-Ile[2], Ile[4]]arginine vasopressin[29] into a single molecule. We hoped that this combination would preserve the oxytocic activity but that the interaction of oxytocin with V_2-receptors leading to antidiuresis would be blocked. More specifically, we assumed that due to dissociation of the chimera into one molecule of oxytocin and one molecule of an antagonist of the vasopressin V_2-receptor, a potent agent with prolonged diuretic and natriuretic actions could be obtained. Our experiments reported herein show that the overall design principle is valid.

Results and Discussion

Synthesis of Dimers. A previously described strategy[19] for management of peptide thiols and controlled formation of two disulfide bridges was followed, with certain modifications (Scheme 2). Automated stepwise solid-phase chemistry using the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group for N^{α}-amino protection, as well as tris(alkoxy)benzylamide (PAL) anchoring with commercially available poly(ethylene glycol)−polystyrene (PEG-PS) supports, was used to assemble the linear sequences of oxytocin and the vasopressin V_2-antagonist. Couplings were mediated primarily by *N,N*-diisopropylcarbodiimide (DIPCDI) in the presence of 1-hydroxybenzotriazole (HOBt) in *N,N*-dimethylformamide (DMF); 4 equiv each of Fmoc-protected amino acid, coupling reagent, and additive were used with respect to the initial loading of resin-bound amine functionality. However, couplings of Gln and Asn (side chain unprotected) were achieved with the respective pentafluorophenyl (Pfp) active esters in the presence of HOBt (4 equiv each), and a mixture of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), and *N,N*-diisopropylethylamine (DIEA) (4 equiv each) was used to mediate the manual introduction of the N-terminal residue. Three *S*-protecting groups, *S*-(*N*-methyl-*N*-phenylcarbamoyl)sulfenyl (Snm), *S*-acetamidomethyl (Acm), and/or *S*-triphenylmethyl (Trt), were incorporated in various combinations at two positions of the sequences, so that their placement in the orthogonal protection scheme would define the final orientations of the two chains in the target heterodimer. Pmp(Snm)-OH, the required Snm-protected derivative[30] of 1-β-mercapto-β,β-cyclopentamethylenepropionic acid (Pmp), was prepared in good yield (43−56%) from either Pmp(Acm)-OH or Pmp(Meb)-OH by reacting with (chlorocarbonyl)sulfenyl chloride followed by *N*-methylaniline. The remaining building blocks were commercially available or made by published procedures.[20]

Syntheses of the heterodimers, both parallel and antiparallel (**I** and **II**), relied on appropriate linear nonapeptide amide monomer intermediates (Scheme 2). Three peptide-resins were created: the vasopressin V_2-antagonist sequence was synthesized once with internal *S*-Trt and N-terminal Pmp(Acm) and a second time with internal *S*-Acm and N-terminal Pmp(Snm), while the oxytocin was synthesized with internal *S*-Acm and N-terminal Boc-Cys(Snm). Upon acidolytic cleavage/deprotection to release these peptides from the supports, *S*-Acm or *S*-Snm moieties are retained, while *S*-Trt is removed to furnish a free thiol function. The V_2-antagonist sequence was required with either the internal Cys or the N-terminal Pmp group as the free thiol (see below); the former intermediate was obtained directly when the *S*-Trt group was used at the internal Cys and the latter formed in two stages based on reductive conversion of an *S*-Snm group. The key

Scheme 2[a]



[a] See Scheme 1 for explanation of nomenclature. Products and intermediates have Roman numerals in the same order as text mention.

dimerization steps were carried out in solution, and they involved attack by the nucleophilic, liberated thiol of one peptide onto an electrofugal *S*-Snm moiety on a separate chain. The aforementioned directed reactions were generally rapid and effective in mixed aqueous media at pH 8 and occurred with minimal formation of undesired side products. The resultant intermolecular disulfide-linked chimeric intermediates were then treated with iodine to co-oxidize Cys(Acm) residues on adjacent chains.

Initial studies to create the parallel heterodimer (**I**) focused on the directed reaction of the free thiol of Cys[1] of the oxytocin chain with the Snm group of a Pmp residue on the vasopressin V_2-antagonist chain. Surprisingly, the expected linear disulfide-linked heterodimer was not formed; instead, the major observed product was the disulfide dimer of [Cys[6](Acm)]oxytocin, presumably due to air oxidation of the corresponding monomeric species. It is plausible that no mixed disulfide formed because of steric hindrance from the six-membered ring of Pmp. This assumption was consistent with simpler model reactions, carried out under a wide range of conditions both in solution and on the solid phase, which revealed that the Snm group on Pmp was resistant to displacement by thiols (see Supporting Information). Consequently, the strategy to form the heterodimer was reversed with respect to the sulfur protecting groups. Thus, we examined attack by a free thiol group from a Pmp residue of the vasopressin V_2-antagonist (**III**) onto an *S*-Snm group of the N-terminal cysteine residue of the oxytocin monomer (**IV**). To create the required Pmp residue with a free thiol, the corresponding peptide-resin with N-terminal Pmp(Snm) was reduced with dithiothreitol (DTT, 20 equiv) in the presence of Et_3N (20 equiv) in DMF for 1 h, followed by cleavage from the support. This method has the advantage that excess DTT can be removed readily by simple filtration and washing. An alternative way to access the same species was to cleave the peptide first, then dilute the TFA solution with methanol, and add Zn metal to promote reduction (details in Supporting Information).

Given the new protection strategy, the desired direct reaction was indeed successful (Figure 1A,B), and the heterodimeric intermediate (**V**) with one disulfide bridge between the N-terminal Pmp residue of the V_2-antagonist and the corresponding N-terminal Cys residue of oxytocin was isolated by preparative HPLC and oxidized further with iodine in HOAc–H_2O (4:1) to give the

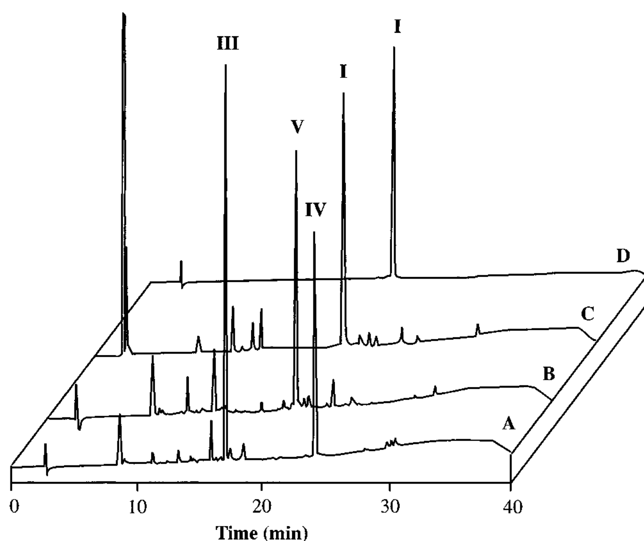


Figure 1. Analytical HPLC evaluation of intermediates **I**–**V**.

target heterodimer (**I**) containing two disulfide bridges (Figure 1C). The overall yield after preparative HPLC purification was 25% (see Figure 1D for analytical HPLC evaluation). When intermediate **V** was oxidized by iodine without first carrying out a purification, the yields were much lower.

Synthesis of the antiparallel heterodimer (**II**) followed a similar strategy. The directed reaction to form the first disulfide bridge involved attack of a free thiol group from the internal Cys^6 residue of the vasopressin V_2-antagonist monomer (**VI**) [which also had N-terminal Pmp(Acm)] onto the N-terminal Cys(Snm) of oxytocin (**VII**) [which also had internal Cys(Acm)]. This step went very smoothly, in reasonable yield and purity. Next, the iodine oxidation step was carried out, to provide the desired dimeric product as the main component detected by analytical HPLC. Preparative HPLC resulted in the isolation of the dimer, albeit in a disappointingly low overall yield of 1.1%.

There can be no doubt that the desired **II** was indeed made. This was proven by a doping experiment with **I**, which showed that these two heterodimers were distinct entities upon HPLC analysis. One possibility that was considered to explain the isolated yield was that the iodine oxidative reaction had not proceeded for a sufficiently long time. However, no improvements were observed upon increasing reaction times 2-, 10-, or 20-fold (data not shown). Steric hindrance of the Pmp residue presumably inhibits the desired intramolecular co-oxidation to close the peptide cycle and allows competing scrambling to occur. Intramolecularly disulfide-cyclized monomers of both oxytocin and the vasopressin V_2-antagonist were indeed observed, and oligomers (not detected by HPLC) could also form leading to a reduced overall yield.

Biological Studies. Chimeras **I** and **II**, prepared as just described, were tested in the classical assays for neurohypophyseal hormones, and the measured activities were compared to values determined for the individual components (Table 1). In the uterotonic test, activities were somewhat lower than was expected from prior activity data on parallel and antiparallel oxytocin homodimers. The effect starts immediately after administration, and hardly any prolongation was observed at the doses applied. These results may be explained by the antiuterotonic activity of the V_2-antagonist. As expected, neither chimera has measurable pressor activity.

Effects of these compounds on diuresis and natriuresis were tested using conscious male rats either without a water load or with a 4% water load. The dynamics of urine excretion was followed every 15 min for 5 h. The quantity of urine excreted during the entire 5-h period was recorded, and the total sodium and potassium in the urine was determined. Finally, for experiments with a starting water load (4%), the diuresis half-time, defined as the time at which one-half of the original load is excreted, was calculated. Once the optimal effective dose for the chimeras was established, i.e., 50 nmol/kg, the relevant parameters were determined using the same dose of oxytocin alone, V_2-antagonist alone, and an equimolar mixture of oxytocin and V_2-antagonist (Table 2).

Results from the diuresis test indicate that the activities of the parallel and antiparallel chimeric peptides are comparable to the activity of the monomer V_2-antagonist; i.e., in the dosage range 2.5–100 nmol/kg, the quantity of urine excreted is approximately the same and the chimeras do not display initial antidiuresis as is the case for oxytocin itself. Furthermore, the effect is somewhat more pronounced for the parallel chimera over the antiparallel one.

Excretion of Na^+ ions is different depending on whether the rats are hydrated. For rats with a water load, the level of ion excretion promoted by chimeras is more or less equal to that of the equimolar mixture of oxytocin and V_2-antagonist, i.e., lower than that of oxytocin alone and higher than that of V_2-antagonist alone. Oxytocin alone causes much higher excretion of sodium. Thus, in the water load case, the effects of oxytocin and V_2-antagonist were *not* additive. These results could be explained by insufficient selectivity of the vasopressin analogue just for the V_2-receptor. However, when nonhydrated rats were used, the parallel chimeric dimer showed the best parameters. The quantity of excreted sodium with the parallel chimera exceeded that of oxytocin alone, V_2-antagonist alone, or a mixture of oxytocin and V_2-antagonist. The quantity of excreted sodium after application of the antiparallel dimer was comparable to that of V_2-antagonist alone, oxytocin alone, or the equimolar mixture of both. While we hypothesize that effects of the dimers are displayed after dissociation, the observed differences between parallel and antiparallel dimers could be explained by different kinetics of dissociation of the dimers. However, no direct experimental data bearing on this issue are available. The possibility of direct interaction of the chimeras with the receptors has not been directly excluded and is in fact consistent with the immediate effect and lack of prolongation in the uterotonic test.

Perspective and Conclusions

One of the goals of the present study was to determine whether one could use disulfide bridging to tailor chimeric molecules that would combine in a single dimeric species defined properties of the corresponding monomers. Based on the hypothesis that the dimers function after their dissociation into monomers, it

Table 1. Biological Activities of Compounds[a]

	activities				
	uterotonic			antidiuretic	
peptides	in vitro (no Mg^{2+})	in vivo	pressor	anesth rats	conscious rats
oxytocin[b]	450	450	4.5	5	100%[e]
V_2-antagonist[c]	$pA_2 = 7.47$	$pA_2 = 6.9$	$pA_2 = 6.42$	$pA_2 = 8.04$	inhibn[f]
oxytocin dimer[d]	1.37	13.8	0.5	ND	~10%
parallel dimer oxytocin/V_2-antagonist	0.22	1.1	0	ND	inhibn[f]
antiparallel dimer oxytocin/V_2-antagonist	0.8	0.8	0	ND	inhibn[f]

[a] Assays and data analysis summarized in the Experimental Section. Unless indicated otherwise, the units of activity are IU/mg; ND, not determined. [b] Literature values from ref 3; used to standardize remaining experiments. [c] The pA_2 values are taken from ref 6 and represent the negative logarithm (base 10) of the concentration (in M) required for half-inhibition of oxytocin activity. [d] As described in ref 19, a mixture of parallel and antiparallel homodimers was isolated as a byproduct from synthetic efforts directed at certain heterodimers. Activity data are also repeated from ref 19, except for antidiuretic activity in conscious rats (this study). [e] Relative to the activity of oxytocin alone, which is defined as 100%. [f] All three compounds with this label have the same degree of inhibitory effects (antagonism) at similar doses.

Table 2. Effects of Various Compounds on Diuresis Half-Time, Volume of Excreted Urine, and Monovalent Cation Content in Urine[a]

	diuresis half-time (min)	vol of excreted urine in 5 h (mL)		total Na^+ (μmol)		total K^+ (μmol)	
peptides	water load 4%	water load 0%	water load 4%	water load 0%	water load 4%	water load 0%	water load 4%
physiological saline control	65 ± 5	1.8 ± 0.4	8.3 ± 0.5	72 ± 29	71 ± 14	79 ± 21	82 ± 16
oxytocin	178 ± 5	4.8 ± 0.3	11.3 ± 0.4	563 ± 15	773 ± 78	202 ± 17	287 ± 28
V_2-antagonist	54 ± 2	9.8 ± 0.1	14.2 ± 0.9	431 ± 49	275 ± 41	220 ± 26	152 ± 24
equimolar mixture of oxytocin and V_2-antagonist	41 ± 5	8.1 ± 1.0	14.1 ± 0.7	480 ± 81	476 ± 20	194 ± 17	196 ± 25
parallel chimeric dimer	42 ± 2	9.4 ± 0.7	14.2 ± 0.8	762 ± 79	375 ± 71	217 ± 27	162 ± 29
antiparallel chimeric dimer	49 ± 2	6.3 ± 0.6	13.1 ± 0.7	358 ± 39	480 ± 30	200 ± 30	123 ± 10
antiparallel oxytocin homodimer[b]	208 ± 10		6.4 ± 1.3		1146 ± 181		231 ± 45

[a] All compounds were tested at the same dose, i.e., 50 nmol/kg of animal. Compounds were administered subcutaneously, and values are expressed as average ± standard error of mean. [b] The oxytocin dimer was also used at the 50 nmol/kg dose. Thus, for proper comparison to other data in the table, it should be noted that after dissociation, this would give 100 nmol of the monomer (i.e., oxytocin).

follows that properties of the various dimer analogues should depend on properties of the individual components. Our previous work with synthetic oxytocin and deaminooxytocin homo- and heterodimers showed that these compounds exhibit prolonged in vivo activity in the uterotonic test. Furthermore, preliminary tests for antidiuretic activity on conscious rats revealed that the oxytocin dimer (present as a mixture of parallel and antiparallel) is active, although the potency is 1 order of magnitude less than that of oxytocin (Table 1). With the goal to attain an agent with both natriuretic and diuretic effects, and without the initial antidiuretic phase, we decided to synthesize chimeric dimers combining into a single molecule oxytocin and a V_2-antagonist. Indeed, the chimeras showed most of the predicted effects, which in most cases were quite close to those of an equimolar mixture of the two individual components. A major exception, which may be of considerable practical importance, occurred in the case of the parallel chimeric peptide dimer when administered to rats without water load. It is known that the diuretic and natriuretic effect of oxytocin depends on the hydration state of the animal.[7] Humans are usually not in a state of overhydration, so the results from our studies are encouraging as a lead toward natriuretic drugs with favorable Na^+/K^+ ratios and minimum side effects.

Somewhat puzzling in light of previous results was the absence of any prolongation effect with the new dimeric compounds. It is possible that the different ways in which peptides were applied may play a role, i.e., intravenous administration for the uterotonic in vivo test versus subcutaneous administration for the antidiuretic test. The difference in time scale—tens of minutes for the uterotonic in vivo test versus hours in the antidiuretic test—may also be significant.

Our concept of using chimeric dimers could be improved by using a more V_2-selective antagonist, together with a more potent natriuretic analogue of oxytocin. The currently chosen antagonist,[29] as a monomer, inhibits in vitro and in vivo uterotonic activities of oxytocin.[29] Hence, this antagonist as a component of the chimera inhibits not only the undesired interaction of the oxytocin component with the V_2-receptors but also the desired interaction with oxytocin receptors. We expect that appropriate iterative redesign and testing of chimeras along the lines described herein could lead to useful active and specific compounds that are diuretic as well as uterotonic and/or natriuretic.

Experimental Section

General. Materials, solvents, instrumentation, and general methods were essentially as described in previous publications from our laboratories.[20,31] Fmoc-PAL-PEG-PS supports (initial loading 0.15−0.18 mmol/g) for peptide synthesis were obtained from the BioSearch Division of PerSeptive Biosystems (Framingham, MA). Protected Fmoc-amino acid derivatives and coupling reagents were from PerSeptive Bioystems or Advanced Chemtech (Louisville, KY), while Pmp derivatives were from Peptide International (Louisville, KY). Boc-Cys(Snm)-OH was prepared and characterized as described previously.[20] TFA, piperidine, DIEA, and NMM were obtained from Fisher (Pittsburgh, PA). Peptide chain assembly was carried out either manually or on a PerSeptive model 9050 continuous-flow peptide synthesizer. Fmoc removal was achieved with piperidine−DMF (1:4, 2 + 8 min). DIPCDI/HOBt- or HATU/HOAt/DIEA-mediated couplings were carried out for 1−2 h using 4 equiv each of Fmoc-amino acids, reagents, and additives over resin-bound amine. Washings between chemical

steps were carried out with DMF and/or CH_2Cl_2. Upon completion of on-resin steps, peptide-resins (~20 mg, except indicated otherwise) were washed with CH_2Cl_2 and then cleaved for 1 h with either reagent B, TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (92:5:1:1:1) (2 mL), or reagent R, TFA−thioanisole−1,2-ethanedithiol−anisole (90:5:3:2) (2 mL). The cleavage mixtures were then filtered and washed with TFA (2 × 2 mL). The combined filtrates were evaporated by bubbling with a stream of N_2. The crude peptides were precipitated with Et_2O−CH_2Cl_2 (10 mL of 20:1 mixture) at 0 °C, collected by low-speed centrifugation, and dissolved in a suitable medium for analytical HPLC.

Amino acid analysis was performed on a Beckman 6300 analyzer with a sulfated polystyrene cation-exchange column (0.4 cm × 21 cm). Peptides were hydrolyzed in 6 N aqueous HCl−propionic acid (1:1 v/v) plus 2 drops of liquefied phenol to prevent degradation of Tyr, for 1 h at 160 °C. Analytical HPLC was performed using a Vydac analytical C-18 reversed-phase column (218TP54; 5 μm, 300 Å; 0.46 × 25 cm) on a Beckman system using Beckman System Gold software. Peptide samples were chromatographed at 1.2 mL/min flow rate, using a linear gradient over 20 min of 0.1% aqueous TFA and acetonitrile (containing 0.1% TFA) from 9:1 in 20 min to 3:2, detection at 220 nm. Purification of crude peptide products was carried out on a Waters Deltaprep 3000 semipreparative HPLC using a Vydac semipreparative C-18 reversed-phase column (218TP1610; 10 μm, 300 Å; 1.0 × 25 cm), elution at 5 mL/min, with a linear gradient of 0.1% aqueous TFA−CH_3CN from 9:1 to 1:4 over 65 min with detection at 220 nm. Fractions with the desired peptide were pooled and lyophilized to provide white powders. The final isolated yields were calculated by comparison of amino acid analyses of the purified peptide to the initial loading of the resin.

^{1}H NMR spectra were observed in the indicated solvents with a Varian VXR 300 instrument. Exchangeable protons are not reported. Low-resolution fast atom bombardment mass spectroscopy (FABMS) was carried out on a VG Analytical 707E-HF low-resolution double-focusing mass spectrometer equipped with a VG 11/250 data system, operated at a resolution of 2000 and 4000. MALDI was performed using a Finnigan FT/MS 2001 instrument. A nitrogen laser was used as the light source (6 mW power, 377 nm wavelength, 3 ns duration), and argon was pulsed into the cell to assist in trapping and cooling of the ions. The matrix used was dihydroxybenzoic acid. A solution of matrix and sample in CH_3CN−H_2O (1:4) was allowed to evaporate on the target. Poly(propylene glycol) was used as the reference material in accurate mass determination.

***N*-Methyl-*N*-phenylcarbamoylsulfenyl-1-*β*-mercapto-*β*,*β*-cyclopentamethylenepropionic Acid [Pmp(Snm)-OH]. A.** A solution of (chlorocarbonyl)sulfenyl chloride[32] (0.7 mL, 8.2 mmol) in $CHCl_3$ (8 mL) was added dropwise at 0−5 °C into an ice bath-cooled suspension of Pmp(Acm)-OH (2.0 g, 8.2 mmol; R_f = 0.29 [$CHCl_3$−HOAc (100:1)]) in $CHCl_3$ (35 mL) over 10 min. After an additional 15 min of stirring, the reaction mixture was filtered and the filtrate was added slowly to a solution of *N*-methylaniline (8.8 mL, 81 mmol) in $CHCl_3$ (25 mL) at 0 °C over 10 min. The reaction mixture was stirred for an additional 30 min and then washed with 1 N aqueous HCl (2 × 80 mL) and H_2O (80 mL). The organic phase was dried (Na_2SO_4), concentrated in vacuo, and purified by flash column chromatography [$CHCl_3$−HOAc (100:1)] to provide the product as a brown crystalline mass. Yield: 1.21 g (43%), mp 151−152 °C, R_f = 0.79 [$CHCl_3$−HOAc (100:1)]. ^{1}H NMR ($CDCl_3$): δ 7.19−7.52 (m, 5H), 3.34 (s, 3H), 2.51 (s, 2H), 1.25−1.77 (m, 10H). FABMS: *m*/*z* calcd for $C_{16}H_{21}NO_3S_2$ 339.10, found 340.10 [$(MH)^+$].

B. Title product was obtained in the same way (1.2-mmol scale), except starting with *S*-4-methylbenzyl-1-β-mercapto-β,β-cyclopentamethylenepropionic acid [Pmp(Meb)-OH] instead of Pmp(Acm)-OH. Yield: 0.23 g (56%).

***S*-(Methyloxycarbonyl)sulfenyl-1-*β*-mercapto-*β*,*β*-cyclopentamethylenepropionic Acid [Pmp(Scm)-OH].** A solution of (chlorocarbonyl)sulfenyl chloride (0.17 mL, 2.0 mmol) in $CHCl_3$ (2 mL) was added dropwise at 0−5 °C into an ice bath-cooled suspension of Pmp(Acm)-OH (0.5 g, 2.0 mmol) in $CHCl_3$ (8 mL) over 5 min. After an additional 15 min of stirring, CH_3OH (20 mL) was added and the solution was stirred for a further 30 min. The reaction mixture was next concentrated in vacuo, and the residue was purified by flash column chromatography to give the product as an off-white solid. Yield: 96 mg (18%), mp 77−84 °C, R_f = 0.44 [$CHCl_3$−CH_3OH−HOAc (30:1:0.1)]. ^{1}H NMR ($CDCl_3$): δ 3.91 (s, 3H), 2.67 (s, 2H), 1.32−2.0 (m, 10H). FABMS: *m*/*z* calcd for $C_{10}H_{16}O_4S_2$ 264.0, found 265.0 [$(MH)^+$].

1-*β*-Mercapto-*β*,*β*-cyclopentamethylenepropionic Acid (Pmp-OH). Pmp(Snm)-OH (0.2 g, 0.59 mmol) was dissolved in CH_3OH (40 mL), and then, TFA (2 mL) and zinc dust (385 mg, 5.9 mmol) were added. The reaction mixture was stirred and monitored by TLC [$CHCl_3$−CH_3OH−HOAc (15:1:0.1)]. After 2 h, the mixture was concentrated in vacuo, and the residue was dissolved in EtOAc (20 mL), washed with H_2O (3 × 20 mL), dried (Na_2SO_4), and concentrated in vacuo to give the product as a white powder. Yield: 58 mg (57%), mp 68−71 °C, R_f = 0.45. ^{1}H NMR ($CDCl_3$): δ 2.66 (s, 2H), 1.30−1.85 (m, 10H). FABMS: *m*/*z* calcd for $C_8H_{14}O_2S$ 174.3, found 175.1 [$(MH)^+$].

Pmp(SH)-D-Ile-Phe-Ile-Asn-Cys(Acm)-Pro-Arg-Gly-NH_2 (III). A. The peptide-resin, Pmp(Snm)-D-Ile-Phe-Ile-Asn-Cys(Acm)-Pro-Arg(Pmc)-Gly-PAL-PEG-PS (30 mg, initial loading 0.15 mmol/g), was treated with DTT (14 mg, 90 μmol) in H_2O−CH_3CN (1:1, 1 mL) for 1 h, with stirring, under an argon atmosphere. The peptide-resin was then washed with DMF and CH_2Cl_2 and cleaved from the resin with reagent R (2 mL). After the workup as described in "General", analytical HPLC revealed the formation of the title product (t_R = 24.2 min, 73%). FABMS: *m*/*z* calcd for $C_{52}H_{85}N_{14}O_{11}S_2$ 1144.4, found 1145.6. Alternative methods involving acidolytic cleavage of the peptide-resin, followed by solution reductions either with zinc in the mixture of CH_3CN, H_2O, and CH_3OH or with aqueous DTT−Et_3N, were also studied (see Supporting Information).

Parallel Heterodimer of Oxytocin and V_2-Antagonist (I). The starting peptides for the procedure were obtained upon cleavage and workup of the corresponding peptide-resins (Scheme 2), as described in "General", and quantified by amino acid analyses and analytical HPLC peak areas. A solution of Pmp(SH)-D-Ile-Phe-Ile-Asn-Cys(Acm)-Pro-Arg-Gly-NH_2 (**III**; 5 μmol, t_R = 24.2 min) in H_2O−CH_3CN (1:1; 10 mL) and a solution of H-Cys(Snm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2 (**IV**; 5 μmol, t_R = 17.0 min) in H_2O−CH_3CN (1:1; 3.1 mL) were combined and mixed with 0.1 M phosphate buffer, pH 8 (2 mL). The resultant homogeneous mixture was stirred under an argon atmosphere; formation of the linear single disulfide heterodimer **V** (t_R = 19.6 min) was monitored by analytical HPLC. After 1.5 h reaction, the intermediate was purified by preparative HPLC and lyophilized. The intermediate was then dissolved in H_2O−CH_3CN (1:1; 2 mL) and combined with a solution of I_2 (25 mg, 0.1 mmol, 20 equiv) in HOAc (47 mL). After stirring for 1.5 h, H_2O (50 mL) was added, and the excess I_2 was removed by extraction with CCl_4 (4 × 40 mL). The aqueous phase was lyophilized, and the title peptide (t_R = 20.0 min) was purified by preparative HPLC (see "General"). Yield: 5.2 mg (25%, based on initial loading). MALDI-MS: *m*/*z* calcd for $C_{92}H_{143}N_{25}O_{22}S_4$ 2077.97, found 2079.0 [$(MH)^+$]. Amino acid composition: Asx, 2.27; Glx, 1.06; Pro, 2.19; Gly, 2.23; Ile, 2.44; Leu, 1.03; Tyr, 0.86; Phe, 0.89.

Antiparallel Heterodimer of Oxytocin and V_2-Antagonist (II). Following a similar procedure as above, Pmp(Acm)-D-Ile-Phe-Ile-Asn-Cys(SH)-Pro-Arg-Gly-NH_2 (**VI**; 19 μmol, t_R = 21.4 min) and H-Cys(Snm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2 (**IV**; 19 μmol, t_R = 17.0 min) were dissolved separately in H_2O−CH_3CN (1:1, 3 mL each). The solutions were combined, diluted with phosphate buffer, pH 8 (3 mL), and stirred under an argon atmosphere. The resultant linear single disulfide heterodimer **VII** (t_R = 20.5 min) was purified by preparative HPLC and lyophilized. The intermediate was then dissolved in H_2O−CH_3CN (1:1, 2 mL), and a solution of I_2 (95 mg, 0.37 mmol) in HOAc (8 mL) was added. After stirring

for 1.5 h, H_2O (38 mL) was added, and the excess I_2 was removed by extraction with CCl_4 (4 × 40 mL). The aqueous phase was lyophilized, and the title peptide (t_R = 19.6 min) was purified by preparative HPLC (see "General"). Yield: 0.85 mg (1.1%). MALDI-MS: *m/z* calcd for $C_{92}H_{143}N_{25}O_{22}S_4$ 2077.97, found 2079.6 [(MH)$^+$]. Amino acid composition: Asx, 2.15; Glx, 0.93; Pro, 1.95; Gly, 2.21; Ile, 2.75; Leu, 0.94; Tyr, 0.59; Phe, 1.06.

Proof That Parallel and Antiparallel Dimers Are Distinct Species. Because antiparallel heterodimer **II**, isolated in low yield as described above, is an isomer of the parallel heterodimer **I**, MALDI-MS alone was not considered to provide conclusive evidence for its structure. Therefore, a co-injection experiment was carried out. Using a Vydac C_4 analytical column, an approximately equimolar mixture of **I** and **II** was chromatographed at 1.0 mL/min flow rate, under isocratic conditions of 0.1% aqueous TFA and 0.1% TFA in acetonitrile (7:3) over 25 min, detection at 218 nm; **I** (t_R = 15.2 min) and **II** (t_R = 14.5 min) were baseline separated.

Biological Evaluation.[33] Wistar rats were used in all experiments. Female rats were estrogenized 24−48 h before the experiment. The uterotonic test was carried out in vitro in the absence of magnesium ions[34,35] and also in vivo.[36] The vasopressor test was performed using phenoxybenzamine-treated male rats.[37] Synthetic oxytocin was used as a standard in uterotonic tests, and synthetic arginine vasopressin was used in pressor tests. Dose−response (single administration) or cumulative dose−response (measurements without washing steps between administration of enhanced doses) curves were constructed. The activities were determined by comparing the threshold doses of the standard and the analogues. Values reported (Table 1) are averages of 3−5 separate experiments.

Tests to estimate antidiuretic, diuretic, and natriuretic properties were conducted on conscious male rats in two variations of the Burn[38] test, as modified.[39] In the standard way with hydrated rats, the animals fasted for 16 h were weighed and then given tap water by means of stomach catheter. The water load was 4% of body weight. Immediately after the water load, the tested substances (or physiological saline as a control) were applied subcutaneously in doses of 1−100 nmol/kg. The rats were then placed into individual metabolic cages, and their urine was collected over a 5-h period. Each day of the experiment, 21 rats were used, divided into 5 groups of 4 or 5 rats getting different doses and compounds; each dose was tested in 2 or 3 independent experiments (different days, different rats). To test for effects with nonhydrated rats, no water load was given to the fasting animals. The concentration of sodium and potassium ions in the rat urine was determined using an Eppendorf model FCM 6341 flame spectrophotometer.

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Supporting Information Available: Experimental descriptions of model studies and routes that failed or gave less-than-optimal results. This material is available free of charge via the Internet at http://pubs.acs.org.

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