anxiolytics, flurazepam is a hypnotic, and clonazepam is an antiepileptic. Fourteen additional 1,4-benzodiazepine derivatives are marketed outside the U.S., many of them under several trade names.

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[1-Penicillamine,2-leucine]oxytocin. Synthesis and Pharmacological and Conformational Studies of a Potent Peptide Hormone Inhibitor[1]

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[1-Penicillamine,2-leucine]oxytocin was synthesized by the solid-phase method of peptide synthesis and purified by partition chromatography on Sephadex G-25, followed by gel filtration. The peptide was found to be a very potent competitive inhibitor of oxytocin in the oxytocic assay with a pA_2 of 7.14 and an inhibitor of oxytocin in the milk-ejecting assay. The compound showed no agonist activity in either of these assays, and its inhibitory activity at the uterus was of prolonged duration. The ^{13}C nuclear magnetic resonance spectral properties and the ^{13}C T_1 (spin–lattice) relaxation times of [Pen^1,Leu^2]oxytocin were determined, and the results were compared with previous studies of [Pen^1]oxytocin, a related competitive inhibitor, and oxytocin, the native hormone agonist. These studies indicated that the hormone inhibitors [Pen^1,Leu^2]oxytocin and [Pen^1]oxytocin have similar conformational and dynamic properties which are different than those of the agonist, oxytocin.

Peptide hormone competitive inhibitors (antagonists) constitute a potentially useful class of organic compounds in clinical applications and for studying peptide–receptor interactions and the mechanisms of peptide hormone action. These applications derive from their ability to interact with the receptor in a manner similar to the hormone and their inability to transduce a biological message to effect a change in the target cells metabolism or other properties. Thus a peptide hormone competitive inhibitor can provide information of the hormone–receptor interaction independent of the transduction event and important clues to structural and dynamic features related to both binding and transduction.

Recently we have shown that [1-penicillamine]oxytocin ([Pen^1]oxytocin, S-C(CH$_3$)$_2$CH(NH$_2$)CO-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH$_2$), a competitive inhibitor of oxytocin,[3,4] has considerably restricted dynamic properties relative to those of oxytocin.[5,6] These studies suggested that certain specific differences in the conformational, dynamic, and structural properties of the hormone and antagonist were related to differences in biological activity.[6]

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A number of half-penicillamine-1 and related analogues of oxytocin have been shown to be potent competitive inhibitors of oxytocin in the in vitro rat uterus oxytocic assay[7] and in other assays. To further our understanding of the structural, dynamic, and conformational properties of half-penicillamine-1 analogues of oxytocin which make them hormone antagonists, we report here the solid-phase synthesis of [Pen[1],Leu[2]]oxytocin and its highly potent inhibitory activities in the oxytocic and milk-ejecting assay systems. In addition, ^{13}C nuclear magnetic resonance (NMR) chemical shift and spin–lattice relaxation (T_1) data are given, which, together with previous circular dichroism (CD) and lasar Raman studies,[17] provide further insight into the conformational and dynamic properties of these hormone antagonists.

The leucine-2 analogue was chosen because, from a structural point of view, it should have similar hydrophobic and steric properties to the tyrosine residue and hence not significantly affect the conformation. A second important structural property is that [Pen[1],Leu[2]]oxytocin no longer contains an aromatic nucleus which can interfere in the interpretation of CD (especially the disulfide chromophore) and other physical data. From the biological point of view, this analogue also should be of interest. NMR data indicated[5,6] that the conformational and dynamic properties of the residue in position 2 of oxytocin and [Pen[1]]oxytocin (i.e., Tyr) were different, but little has been done to explore the structural requirements in the 2 position in relation to inhibitory activities of [Pen[1]]-oxytocin analogues. Interestingly, several 2-position substituted oxytocin analogues are inhibitors. It was already known that [Leu[2]]oxytocin is an agonist,[8] and thus we expected [Pen[1],Leu[2]]oxytocin to be an antagonist. Indeed the latter compound was determined to be one of the most potent inhibitors found thus far in the oxytocic assay.

The required precursor peptide to [Pen[1],Leu[2]]oxytocin, Cbz-Pen(Bzl)-Leu-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2, was synthesized by the solid-phase method of peptide synthesis[9] according to procedures generally used in our laboratory for the synthesis of oxytocin derivatives and analogues.[10,11] The protecting groups were removed with sodium in liquid ammonia and the sulfhydryl compound was oxidized with 0.01 N $K_3Fe(CN)_6$[12] to the disulfide product. The compound was purified by partition chromatography on Sephadex G-25 using the solvent system 1-butanol–3.5% aqueous HOAc containing 1.5% pyridine (1:1), followed by gel filtration on Sephadex G-25 using 0.2 N HOAc as the eluant solvent. A solid-phase synthesis of [Pen[1]]oxytocin was also carried out (see Experimental Section) for comparison purposes and to obtain the compound for comparative pharmacological and biophysical studies. The purity of the compounds was assessed by their chromatographic behavior, thin-layer chromatography in three solvent systems, optical rotation, quantitative amino acid analysis, and elemental analysis.

[Pen[1],Leu[2]]oxytocin was assayed for its oxytocic and milk-ejecting activities (see Experimental Section), and the biological activities were compared to those of [Leu[2]]-oxytocin and [Pen[1]]oxytocin and other oxytocic inhibitors. [Pen[1],Leu[2]]oxytocin had no oxytocic activity. However, the compound was found to have a potent inhibitory effect on the oxytocic response to oxytocin. The antioxytocic potency of [Pen[1],Leu[2]]oxytocin was found to be quite high with pA_2 of 7.14 ($M = 7.3 \times 10^{-8}$; SD = 1.4×10^{-8}; $n = 12$). This makes it substantially more potent than [Pen[1]]-oxytocin and places it among the most potent oxytocic inhibitors known as shown in Table I. At a weight/weight ratio ([Pen[1],Leu[2]]oxytocin–oxytocin) of 20:1, a 50% inhibition of the oxytocin response (the first induced contraction) was observed when the antagonist was injected 10–20 s prior to the agonist. A longer prior contact time of up to 90 s with [Pen[1],Leu[2]]oxytocin did not significantly affect the inhibitory activity. The inhibitory effect was readily reversible. After washout, the oxytocic response to the next injection of oxytocin returned nearly completely or completely to the control level.

Some evidence that [Pen[1],Leu[2]]oxytocin may have slightly slower association and dissociation rates than oxytocin was suggested by the following experiments shown in Figure 1. In these experiments, the time course of the oxytocic response was followed for a given interval of time under various conditions. Panel A of Figure 1 shows the uterine contractions in response to a 6-min exposure to a submaximal dose (2.5 mU/mL) of oxytocin. Regular phasic contractions of the uterus were observed during the time interval of drug exposure. In panel B, [Pen[1],Leu[2]]oxytocin (0.25 μg/mL) was added 30 s prior to oxytocin. An initial oxytocin response (about 60% inhibited) was observed but none of the subsequent contractions occurred during the 10-min interval of drug exposure. When these same doses of [Pen[1],Leu[2]]oxytocin and oxytocin were injected simultaneously, little or no inhibition of the oxytocin response was seen. Regular phasic contractile response to oxytocin was maintained during the 6-min interval of drug exposure. These observations suggest that [Pen[1],Leu[2]]oxytocin has a lower receptor-binding affinity than oxytocin. However, once bound to the receptor, [Pen[1],Leu[2]]oxytocin apparently is not readily displaced by the agonist oxytocin (i.e., it has a slower dissociation rate). The inhibition, however, is

Table I. Agonist and Inhibitor Oxytocin Analogues in the Oxytocic Assay

compd	oxytocic inhibitory act., pA_2[a]	oxytocic act. (USP), U/mg
oxytocin		546 ± 18[b]
[Pen[1]]oxytocin	6.86[c]	
[β-MPA[1]]oxytocin		803 ± 36[d]
[β-MPA(β-Me_2)[1]]oxytocin	6.94[c]	
[β-MPA(β-Et_2)[1]]oxytocin	7.24[c]	
[β-MPA(β-Me_2)[1],Thr[4]]oxytocin	7.50[e]	
[β-MPA[β-$(CH_2)_5$][1]]oxytocin	7.43[f]	
[Leu[4]]oxytocin		13 ± 1[g]
[Pen[1],Leu[4]]oxytocin	6.77[h]	
[β-MPA(β-Et_2)[1],Leu[4]]oxytocin	7.02[i]	
[β-MPA[1],Dbt[2]]oxytocin	7.05[j]	
[β-MPA(β-Et_2)[1],Dbt[2]]oxytocin	7.08[k]	
[Leu[2]]oxytocin		0.5[l]
[Pen[1],Leu[2]]oxytocin	7.14	

[a] pA_2 values represent the negative log to the base 10 of the average molar concentration ($\overline{M}$) of an antagonist which will reduce the response of the uterine horn of $2x$ units of pharmacologically active compound (agonist) to x units of the agonist. [b] W. Y. Chan, M. O'Connell, and S. R. Pomeroy, *Endocrinology*, 72, 279 (1963). [c] R. J. Vavrek, M. F. Ferger, G. A. Allen, D. H. Rich, A. T. Blomquist, and V. du Vigneaud, *J. Med. Chem.*, 15, 123 (1972). [d] B. M. Ferrier, D. Jarvis, and V. du Vigneaud, *J. Biol. Chem.*, 240, 4264 (1965). [e] J. J. Nestor, Jr., M. F. Ferger, and V. du Vigneaud, *J. Med. Chem.*, 18, 284 (1975). [f] M. Manning, J. Lowbridge, J. Seto, J. Halder, and W. H. Sawyer, *ibid.*, 21, 179 (1978). [g] V. J. Hruby, G. F. Flouret, and V. du Vigneaud, *J. Biol. Chem.*, 244, 3890 (1969). [h] M. Ferger and W. Y. Chan, *J. Med. Chem.*, 18, 1020 (1975). [i] D. F. Dyckes, J. J. Nestor, Jr., M. F. Ferger, V. du Vigneaud, and W. Y. Chan, *ibid.*, 17, 969 (1974). [j] Lundell et al.[18] [k] Lundell et al.[19] [l] Hruby et al.[8]

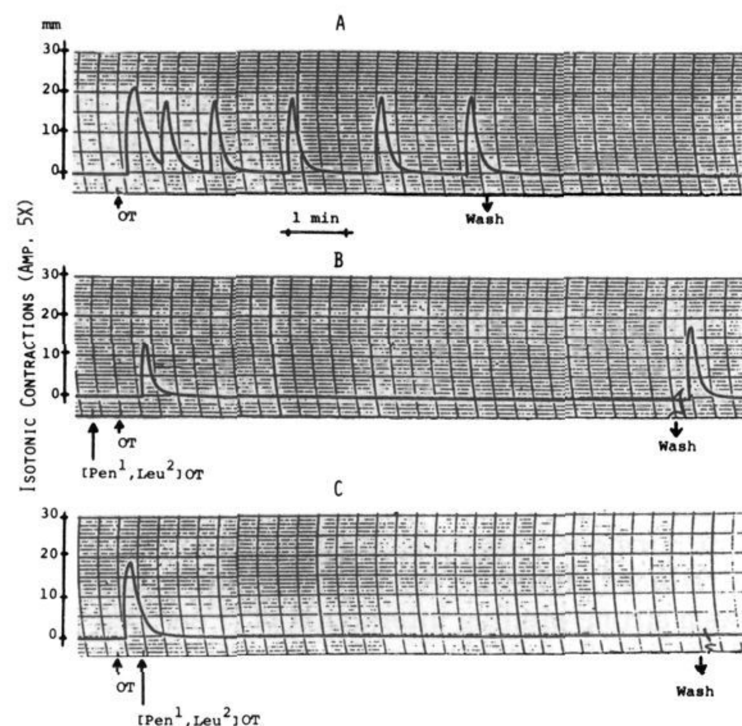

Figure 1. Inhibition of oxytocin response by [Pen[1],Leu[2]]oxytocin in an isolated rat uterus. All injections were allowed to remain in the organ bath for the duration indicated. Up arrows indicate points of injections. Down arrows indicate draining and rinsing of the organ bath. Panel A, oxytocin (OT), 2.5 mU/mL; panel B, [Pen[1],Leu[2]]oxytocin, 0.25 μg/mL, followed in 20 s by oxytocin, 2.5 mU/mL; panel C, oxytocin, 2.5 mU/mL, followed by [Pen[1],Leu[2]]oxytocin, 0.25 μg/mL, injected at the peak of the first contraction induced by oxytocin; estrous uterus; isotonic contraction recording; bath temperature 30 °C.

completely reversible. As in the single contraction and washout assay system, the oxytocin response following the washout always returned to, or nearly to, its control level. Interestingly, when the same dose of [Pen[1],Leu[2]]oxytocin was injected at the peak of the first contraction induced by oxytocin, as shown in panel C of Figure 1, no subsequent oxytocin-induced contractions were observed during the 10-min interval of drug exposure. It is worthy to note that in the two cases where [Pen[1],Leu[2]]oxytocin produced the inhibitory effect, the antagonist was introduced either when the oxytocin concentration was zero or declining relative to the antagonist and the uterus was in a quiescent state or entering the relaxation phase. Apparently conditions are more favorable for the *antagonist* under these conditions than when the antagonist and the agonist are introduced simultaneously.

The oxytocic inhibitory effects of [Pen[1],Leu[2]]oxytocin described above were highly reproducible and were consistently demonstrated in all the experiments (three or more in each category) performed.

[Pen[1],Leu[2]]oxytocin showed no milk-ejecting activity (Figure 2) even at concentrations as high as 10^{-5} M. Complete inhibition of oxytocin at 5×10^{-9} M could be seen at a [Pen[1],Leu[2]]oxytocin concentration of 7.5×10^{-6} M (Figure 2). [Pen[1]]oxytocin was found to inhibit the milk-ejecting response to oxytocin, but this analogue was also a partial agonist, making it difficult to quantitate its inhibitory potency in this assay.

The ^{13}C nuclear magnetic resonance spectrum of [Pen[1],Leu[2]]oxytocin was obtained. The assignments were made by comparison of the spectrum of [Pen[1],Leu[2]]-oxytocin with oxytocin,[13] [Pen[1]]oxytocin,[5] and several deuterated derivatives of oxytocin.[14] The empirical rules for shifts of alkanes[15] were used to assign the half-penicillamine-1 carbon resonances. The chemical shifts are given in Table II, along with those for [Pen[1]]oxytocin taken under similar conditions. It is worthy of note that the chemical shifts for all carbon atoms in [Pen[1],Leu[2]]-oxytocin and [Pen[1]]oxytocin except those at the variable

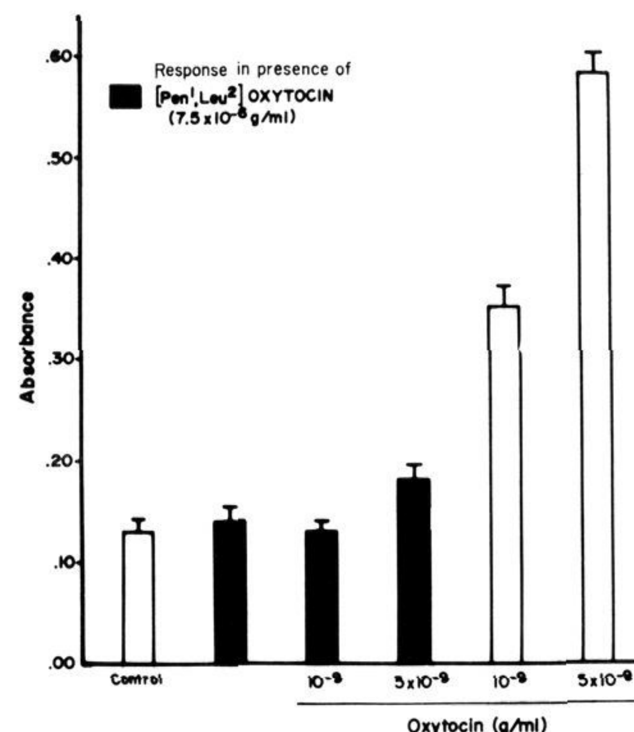

Figure 2. In vitro demonstration of [Pen[1],Leu[2]]oxytocin inhibition of oxytocin-induced milk ejection. Mouse mammary gland tissue was incubated in the presence of oxytocin and also in the presence of oxytocin and the antagonist, [Pen[1],Leu[2]]-oxytocin. Each value represents the mean ± SE response (milk-ejection) of milk released from mammary tissue of four mice as measured spectrophotometrically (absorbance).

Table II. ^{13}C Chemical Shifts of [Pen[1],Leu[2]]oxytocin and [Pen[1]]oxytocin in D_2O at 30 °C, pH 3.8[a]

	carbon atom	[Pen[1],-Leu[2]]-oxytocin	[Pen[1]]-oxytocin
half-Pen[1]	α-CH	62.0	62.0
	β-C	51.7	51.6
	γ-CH_3	26.4	26.7
	γ-CH_3	26.0	26.7
Leu[2]	α-CH	54.0	
	β-CH_2	40.5	
	γ-CH	25.3	
	δ-CH_3	23.3	
	δ-CH_3	21.7	
Tyr[2]	α-CH		56.6
	β-CH_2		36.3
	C-1′		127.7
	C-2′,6′		130.8
	C-3′,5′		116.2
	C-4′		154.5
Ile[3]	α-CH	61.3	60.6
	β-CH	37.1	36.7
	γ-CH_2	25.5	25.7
	γ-CH_3	15.8	15.5
	δ-CH_3	11.9	12.0
Gln[4]	α-CH	56.5	56.3
	β-CH_2	26.3	27.0
	γ-CH_2	31.7	32.1
Asn[5]	α-CH	51.4	51.2
	β-CH_2	40.6	40.7
half-Cys[6]	α-CH	53.0	52.7
	β-CH_2	38.0	37.9
Pro[7]	α-CH	61.8	62.0
	β-CH_2	30.3	30.4
	γ-CH_2	25.8	26.1
	δ-CH_2	49.0	49.0
Leu[8]	α-CH	53.7	53.5
	β-CH_2	40.5	40.5
	γ-CH	25.5	26.1
	δ-CH_3	23.3	22.7
	δ-CH_3	21.7	21.7
$GlyNH_2$[9]	α-CH_2	43.2	43.2

[a] Chemical shifts are measured in parts per million from Me_4Si (external) using internal 1,4-dioxane at 67.6 ppm. On this scale Me_4Si (external) is 193.7 ppm upfield from $^{13}CS_2$. The shifts are accurate to 0.1 ppm.

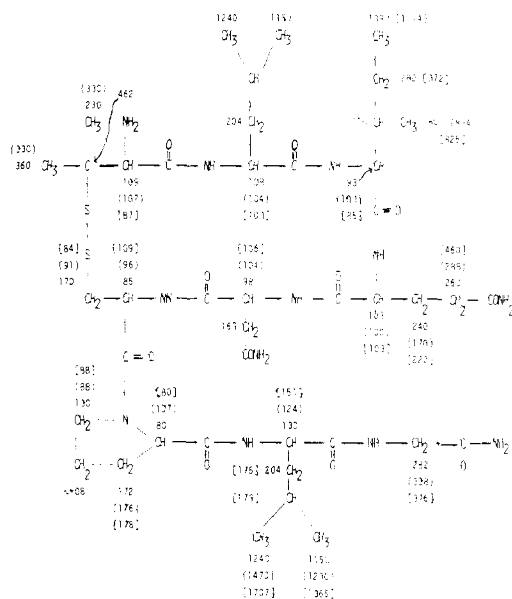

Figure 3. NT_1 values for the aliphatic carbons of [Pen[1],Leu[2]]oxytocin (7.8×10^{-2} M) in D_2O at pH 3.8, 30 °C (open numbers); for [Pen[1]]oxytocin (7.8×10^{-2} M) in D_2O at pH 3.8, 28 °C (numbers in parentheses);[5] and for oxytocin (10×10^{-2} M) in D_2O at pH 3.5, 32 °C (numbers in brackets).[16]

2 position and a few others are essentially identicial. The NT_1 (spin–lattice relaxation) values of individual carbon atoms in [Pen[1]]oxytocin in aqueous solution are given in Figure 3 and are compared with those previously obtained for oxytocin[16] and [Pen[1]]oxytocin.[5] With a few exceptions to be discussed below the NT_1 values obtained are very similar to those of [Pen[1]]oxytocin obtained under very similar conditions.

Previously, a comprehensive investigation of the laser Raman spectra and the CD spectra of these antagonists and other compounds over a variety of pH values was made.[17] From these studies it was concluded that [Pen[1],Leu[2]]oxytocin and [Pen[1]]oxytocin have very similar conformational and dynamic properties which are different from those of oxytocin. For example, these antagonists have a relatively more rigid peptide backbone (at least in the 20-membered ring moiety) than oxytocin and a reasonably fixed disulfide moiety with a disulfide bond angle of about +110°.[17]

These general conclusions are supported by the ^{13}C NMR results reported here. The chemical shifts observed throughout the spectrum are virtually the same for both [Pen[1],Leu[2]]oxytocin and [Pen[1]]oxytocin (Table II). The small chemical shift differences seen at the Ile[3] residue probably reflect the different residues found at position 2 (Leu and Tyr, respectively) in the two compounds. The most interesting difference is that for the Pen[1] γ-methyl groups. In [Pen[1]]oxytocin they had the same chemical shifts,[5] but in [Pen[1],Leu[2]]oxytocin they have different chemical shifts (Table II) indicating that in the latter case they are in chemically distinct environments. In the former case the equivalence was possibly a fortuitous result of the differential anisotropic effects of the adjacent tyrosine aromatic moiety. The comparative NT_1 values (Figure 3) for [Pen[1],Leu[2]]oxytocin, [Pen[1]]oxytocin, and oxytocin again indicate that the dynamic properties of the two antagonists are nearly identical with similar restrictions in amino acid side-chain groups and in the tripeptide "tail" but are somewhat different than oxytocin. This is especially clear when comparing the T_1 values at the Gly[9] C-α and various side-chain carbons, e.g., the Gln γ-CH_2, Ile γ-CH_3, and Leu[8] α-CH and δ-CH_3, for the two antagonists relative to those found for oxytocin. The NT_1 and chemical shift values of the Pen[1] γ-CH_3 carbons appear to be slightly different in [Pen[1],Leu[2]]oxytocin. This was not seen in [Pen[1]]oxytocin, but since the chemical shifts were identical for the Pen[1] γ-CH_3 carbon atoms in this compound, it is unlikely that similar differences in the NT_1 values would have been detected.

The conformational and dynamic properties obtained for [Pen[1],Leu[2]]oxytocin provide further evidence for our previous proposals[5] regarding conformational and dynamic properties of oxytocin analogues which can lead to inhibitor activities. [Pen[1],Leu[2]]oxytocin and [Pen[1]]oxytocin have very similar dynamic and conformational properties and thus, as expected, the former compound is also a potent hormone antagonist in the oxytocic assay system. The reduced dynamic flexibility of the antagonist still allows a strong binding interaction of [Pen[1],Leu[2]]oxytocin to the oxytocin receptor in the uterus, but transduction to the biologically active state apparently is prevented, as with [Pen[1]]oxytocin, because element(s) of intrinsic rigidity prevent the formation of the biologically active form of the receptor–hormone complex necessary to induce an agonist response. The trend in NT_1 values of [Pen[1],Leu[2]]oxytocin suggests that it may be somewhat less flexible than [Pen[1]]oxytocin, and perhaps this is related to the more potent antagonist activity of the former compound. The increased potency for [Pen[1],Leu[2]]oxytocin is perhaps somewhat more than expected. With a pA_2 of 7.14 (Table I), the compound is one of the more potent antagonists of oxytocin on the uterus known. Previous investigations have indicated that structural modifications which tend to improve or lower oxytocic activities of agonists also tend to have the same relative effects on inhibitory potencies of [Pen[1]]oxytocin analogues and derivatives (Table I). However, despite the greatly reduced oxytocic agonist activity of [Leu[2]]oxytocin,[8] [Pen[1],Leu[2]]oxytocin is a much more potent antagonist than [Pen[1]]oxytocin. Previous work[18-20] has shown that various position-2 tyrosine derivatives (e.g., 3,5-dibromotyrosine, *o*-methyltyrosine, etc.) can provide analogues with inhibitory activities. However, this report is the first to illustrate that nontyrosine based substitutions in the 2 position of oxytocin can significantly enhance inhibitory activity when used in conjunction with the Pen[1] substituent. These results would suggest that other substituents on the 2 position which would not disrupt the restricted conformational and dynamic properties found in [Pen[1],Leu[2]]- and [Pen[1]]oxytocin should provide even more potent oxytocin antagonists when used in conjunction with the appropriate position-1 residue substituent. The preparation of such derivatives is in progress in our laboratory.

Experimental Section

Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on a silica gel G plates using the following solvent systems: (A) 1-BuOH–HOAc–H_2O (4:1:5, upper phase only); (B) 1-BuOH–HOAc–pyridine–H_2O (15:3:10:12); (C) 1-pentanol–pyridine–H_2O (7:7:6). The load size was 30–60 µg and chromatographic lengths were 120–160 nm. Detection was made by ninhydrin, fluorescamine, and iodine. In all cases, unless otherwise specified, single symmetrical spots were observed for purified materials. Optical rotation values were measured at the mercury green line (547 nm) using a Zeiss Old 4 polarimeter. Amino acid analyses were obtained by the method of Spackman et al.[21] on a Beckman 120C amino acid analyzer following hydrolysis in 6 N HCl for 24 h at 110 °C. Elemental analyses were performed by Spang Microanalytical Laboratory and Heterocyclic Chemical Corp. When analyses are indicated only by symbols of the elements, analytical results for the elements were within ±0.4% of the theoretical value. N^{α}-Boc-protected

Table III

step	normal DCC coupling: solvent or reagent	duration, min	no. of times	nitrophenyl ester coupling (Asn and Gln): solvent or reagent	duration[a]	no. of times
1	CH_2Cl_2	1	4	CH_2Cl_2	1	4
2	TFA-CH_2Cl_2-anisole, 25:73:2	2	1	TFA-CH_2Cl_2-anisole, 25:73:2	2	1
3	TFA-CH_2Cl_2-anisole, 25:73:2	20	1	TFA-CH_2Cl_2-anisole, 25:73:2	20	1
4	CH_2Cl_2	1	3	CH_2Cl_2	1	3
5	DIEA-CH_2Cl_2, 10:90	2	2	DIEA-CH_2Cl_2, 10:90	2	2
6	CH_2Cl_2	1	4	CH_2Cl_2	1	4
7	Boc-AA in CH_2Cl_2 (1.5 equiv)		1	DMF	1	5
8	DCC in CH_2Cl_2 (1.5 equiv)	20	1	Boc-AA in DMF (4 equiv) (4 equiv of HBT)	4-10 h	1
9	CH_2Cl_2	1	2	DMF	1	3
10	EtOH	1	2	EtOH	1	3
11	CH_2Cl_2	1	2			
12	Boc-AA in CH_2Cl_2 (1.5 equiv)		1			
13	DCC in CH_2Cl	20	1			
14	CH_2Cl_2	1	2			
15	EtOH	1	3			

[a] Duration in minutes except when otherwise noted.

amino acids and amino acid derivatives were purchased from Vega-Fox Biochemical or from Peninsula Laboratories. Just before use, all amino acid derivatives were tested for homogeneity by thin-layer chromatography in solvent systems A, B, and C and by mixture melting point determinations with authentic samples. *tert*-Butoxycarbonyl-*S*-3,4-(dimethylbenzyl)cysteine was prepared by published procedures.[22,23] *N*-(Benzyloxycarbonyl)-*S*-benzyl-L-penicillamine was prepared as previously reported.[4] Progress in the condensation reactions was followed by the quantitative ninhydrin test of Kaiser et al.[24] Solvents for partition chromatography were purified as previously reported.[25]

Solid-Phase Synthesis of Cbz-Pen(Bzl)-Leu-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2. The title compound was prepared by the solid-phase method using a semiautomated instrument designed and built in our laboratory.[26] *tert*-Butoxycarbonylglycinate-resin was prepared from polystyrene resin (1% cross-linked with divinylbenzene and chloromethylated to an extent of 1.06 mmol/g of resin; Lab Systems, Inc., San Mateo, CA) using standard procedures.[27] The substitution level of the first amino acid was 0.35 mmol of glycine/g of resin. A 5.7-g (2.0 mmol) sample of this resin was used in the synthesis. The removal of the *N*-Boc-protecting groups, neutralization of the peptide resin salt, and addition of the next amino acid residue to the growing peptide followed the program listed in Table III. For the active ester coupling of asparagine and glutamine, an equivalent molar amount of 1-hydroxybenzotriazole was added to the reaction mixture as a catalyst for the solid-phase coupling. Under these conditions coupling was complete, as judged by the ninhydrin test,[24] after 4 and 10 h for asparagine and glutamine derivatives, respectively. Sixty-milliliter portions of solvent or solution were used throughout the reaction sequence. After each amino acid residue was coupled, the synthesis was monitored for completion of coupling by use of the ninhydrin test, and a negative test was indicated at each step. After completion of the synthesis, the protected peptide resin was washed with CH_2Cl_2 (4 times), EtOH (3 times), DMF (4 times), and CH_2Cl_2 (5 times) and dried in vacuo to give 7.8 g of Cbz-Pen(Bzl)-Leu-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-O-resin.

The protected peptide was cleaved from the resin by adding the resin to a solution of 150 mL of freshly distilled anhydrous methanol which had been saturated with anhydrous ammonia (freshly distilled from Na) at -5 °C. The flask containing the mixture was wired shut and stirred at room temperature for 4 days in a desiccator. The solvents were removed by aspiration and rotary evaporation in vacuo, and the cleaved peptide was extracted from the resin with two 100-mL portions of DMF at 60 °C. The combined organic solvents were evaporated down to about 15 mL in vacuo and water was slowly added. A white flocculent precipitate formed and was filtered off and washed with two 20-mL portions of water, 95% ethanol, and ether. The precipitate was dried in vacuo to give 1.63 g (62%) of Cbz-Pen(Bzl)-Leu-Ile-Gln-Asn-Cys(DMB)-Pro-Leu-Gly-NH_2 as a white powder: mp 239–241 °C dec. Anal. ($C_{66}H_{96}N_{12}O_{13}S_2{\cdot}H_2O$) C, H, N.

[1-Penicillamine,2-leucine]oxytocin. A sample of 337 mg (0.25 mmol) of the protected nonapeptide from above was dissolved in 175 mL of anhydrous NH_3 (freshly distilled from Na) and treated with a sodium stick until a blue color persisted for 60 s. The ammonia was removed by evaporation under nitrogen and the last 15 mL by lyophilization. The white powder was dissolved in 750 mL of deaerated 0.1% aqueous acetic acid under nitrogen. The pH was adjusted to 8.5 with 3 N NH_4OH, and the peptide was oxidized with 50 mL of 0.01 N $K_3Fe(CN)_6$.[12] The pH was adjusted to 4 with 10% aqueous HOAc and the ferro- and excess ferricyanide were removed by addition of Rexyn 203 (Cl^- cycle). After 30 min the resin was filtered off and washed with three 20-mL portions of 10% HOAc. About 75 mL of 1-butanol was added to the combined aqueous solutions, and the mixture was evaporated to about 175 mL in vacuo at 20–30 °C and lyophilized. The product was purified by partition chromatography[28] on Sephadex G-25 (block polymerizate) using the solvent system 1-butanol–3.5% aqueous acetic acid containing 1.5% pyridine (1:1). The fractions corresponding to the product (R_f 0.28) were pooled and isolated as a white powder which was further purified by gel filtration on Sephadex G-25 using 0.2 N HOAc as the eluant solvent. The fractions corresponding to the major peak were pooled and lyophilized to give [Pen^1,Leu^2]-oxytocin as a white powder; 99 mg (40%); $[\alpha]^{25}_{547}$ +11.3° (*c* 0.55, 1 N HOAc). The peptide gave a single uniform spot on thin-layer chromatography using solvents A, B, and C. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.1; Pro, 1.0; Gly, 1.0; half-Cys, 0.36; mixed disulfide of half-Pen and half-Cys, 1.5; Ile, 1.0; Leu, 2.0. Anal. ($C_{42}H_{72}N_{12}O_{11}S_2$) C, H, N.

[1-Penicillamine]oxytocin. The title compound was prepared by the solid-phase methods of peptide synthesis using the same procedures as used in the synthesis of [Pen^1,Leu^2]oxytocin. The nonapeptide Cbz-Pen(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 was obtained as before as a white powder: mp 228–231.5 °C (lit.[3] mp 224–228 °C). A 350-mg (0.25 mmol) portion was converted to the cyclic compound as reported above in the synthesis of [Pen^1,Leu^2]oxytocin. Partition chromatography purification was accomplished using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1). The compound emerged as a single uniform peak at R_f 0.28 (lit.[3] R_f 0.27). Following gel filtration on Sephadex G-25 using 0.2 N HOAc as the eluant solvent, [Pen^1]oxytocin was obtained as a white powder: 91.4 mg (36%); $[\alpha]^{24}_{547}$ +16.1° (*c* 0.54, 1 N HOAc) [lit.[3] $[\alpha]^{20}_D$ +18° (*c* 0.5, 1 N HOAc)]. Thin-layer chromatography with solvent systems A, B, and C gave a single uniform spot identical with authentic [1-penicillamine]oxytocin (we thank Professor du

Vigneaud for a sample of the compound). The compound gave the expected ratios on amino acid analysis. The proton[5,6] and carbon-13[5] nuclear magnetic resonance spectra were consistent with the structure. The compound inhibited the oxytocic activity of oxytocin as previously reported.[3]

Nuclear Magnetic Resonance Spectroscopy. Carbon-13 spin–lattice relaxation measurements (T_1) were made on a Brunker WH-90 FT spectrometer. T_1 data were acquired using a $180° - \tau - 90° - t_\infty$ pulse sequence, where t_∞ is at least five times the longest T_1. The experimental error is ±10–15%. The samples were dissolved in D_2O (99.8%) and the pH was adjusted (direct pH meter readings) to the pH values given with aqueous DCl and NaOD. For the ^{13}C T_1 measurements on [Pen^1,Leu^2]oxytocin a concentration of about 80 mg/mL was used, and samples were degassed and kept under nitrogen at all times. A drop of dioxane was used as the internal standard for all ^{13}C NMR experiments.

Bioassay Studies. Oxytocic assays were performed on isolated uteri from rats in natural estrus as described by Chan et al.[29] with the use of Mg-free van Dyke–Hasting's solution. Antioxytocic potencies were determined by the method of Schild.[30] The pA_2 values represent the negative logarithm to the base of 10 of the average molar concentration ($\bar{M}$) of antagonist which will reduce the biological response of $2x$ units of the agonist to the level of response of x units of agonist. Milk-ejecting activities were measured by the method of Hruby and Hadley.[31]

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References and Notes

(1) All optically active amino acids are of the L variety unless otherwise stated. The abbreviations for the amino acids follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomencalture [*J. Biol. Chem.*, **247**, 977 (1972)]. Other abbreviations include Pen, penicillamine; β-MPA, β-mercaptopropionic acid; DMB, 3,4-dimethylbenzyl; NMR, nuclear magnetic resonance; CD, circular dichroism.

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