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SOLID PHASE SYNTHESIS OF UNSATURATED ANALOGUES OF OXYTOCIN AND THEIR MEDICINAL APPLICATION

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SOLID PHASE SYNTHESIS OF UNSATURATED ANALOGUES OF OXYTOCIN AND THEIR MEDICINAL APPLICATION

by

Khadijeh Kathy Marashi

A Thesis Submitted to the Faculty of the

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ABSTRACT

The preparation of four new oxytocin analogues is described. The synthesis of the protected peptides were performed using solid-phase peptide synthetic methodology. The protected peptides were deprotected and cyclized using anhydrous HF followed by aqueous potassium ferricyanide treatment. The purification of each peptide was accomplished using gel-filtration followed by high-performance liquid chromatography.

The oxytocin analogues prepared in this dissertation are the following: [L-$\Delta^{4,5}$-Leu^{3}]-oxytocin, [L-$\Delta^{4,5}$-Leu^{8}]-oxytocin, [Leu^{2}, Thr^{4}, $\Delta^{3,4}$-Pro^{7}, $\Delta^{4,5}$-Leu^{8}]-oxytocin, [Pen^{1}, Tyr(OMe)^{2}, Thr^{4}, $\Delta^{3,4}$-Pro^{7}, $\Delta^{4,5}$-Leu^{8}]-oxytocin.

These analogues were designed to introduce dehydroamino acids in the binding elements of oxytocin in order to increase uterotonic receptor affinity, and conformational restriction at position 1 and 2 in order to enhance the antagonistic activity of analogues in vivo. Such analogues could be used therapeutically to prevent preterm labor.

CHAPTER 1

INTRODUCTION

Peptide hormones are a ubiquitous class of chemical messengers found throughout the animal kingdom. Their central importance in the control of such diverse biological functions as reproduction, pigmentation, glucose homeostasis, pain perception, growth, water balance, etc. makes them of great importance in understanding the biology and chemistry of complex organisms. Naturally occurring peptides and proteins range in size from small peptides such as the tripeptide glutathione, to proteins with molecular weights of more than 10^6. Their biological activities cover almost as broad a spectrum. Some are relatively inert (e.g., Keratin) while others, such as the polypeptide hormones, are highly active. Unlike proteins however, peptide hormones are more accessible for study because of their ease of preparation, purification, and characterization. Due to the relatively easy accessibility of these compounds, the study of peptide hormone interaction with biological tissues has led to the discovery of the role of these compounds in controlling bodily function and their possible therapeutic applications.

The posterior lobe of the pituitary (neurohypophysis) is unusual in that it, unlike other endocrine organs is under direct nervous

control (Lederis, 1974). Hormones stored in this gland are formed in hypothalamic nerve cells, but pass through nerve stalks into the posterior pituitary. The structures of natural neurohypophyseal hormones are shown in Figure 1. Each compound contains nine amino acids and each has a disulfide bridge between cysteine residues at 1 and 6, variations among naturally occurring nonapeptides are found only in position 2, 3, 4, and 8. The most important structural difference from biological point of view are the changes in the 3 and the 8 position. The relative activities of these compounds, when contrasted with their phyletic distributions and structural variations, suggest an evolutionary relationship among the various peptides (Sawyer, 1977). A representation of the relationship is shown in Figure 2. A common link may be arginine vasotocin which is found in lower vertebrates as well as in the most complex mammals. The most extensively studied biological activities of the neurohypophyseal hormones are given in Table 1. In general, we can divide these biological assays into those concerned primarily with oxytocin-like activities (rat uterus, avidin vasopressor, and milk-ejecting) and those associated with vasopressin-like activities (antidiuretic and pressor). It should be noted that with the exception of arginine-vasotocin (AVT) which possess potent agonist activities at all these receptors, most of the native neurohypophyseal peptides fall into one or other of these classes, that is oxytocin-like (OT, MT, VT, AT, IT, GT) or vasopressin-like (AVP, LVP, PP); generally the relative differential activity is a 100 to 1000 fold difference in activity at these different receptor subtypes. The major

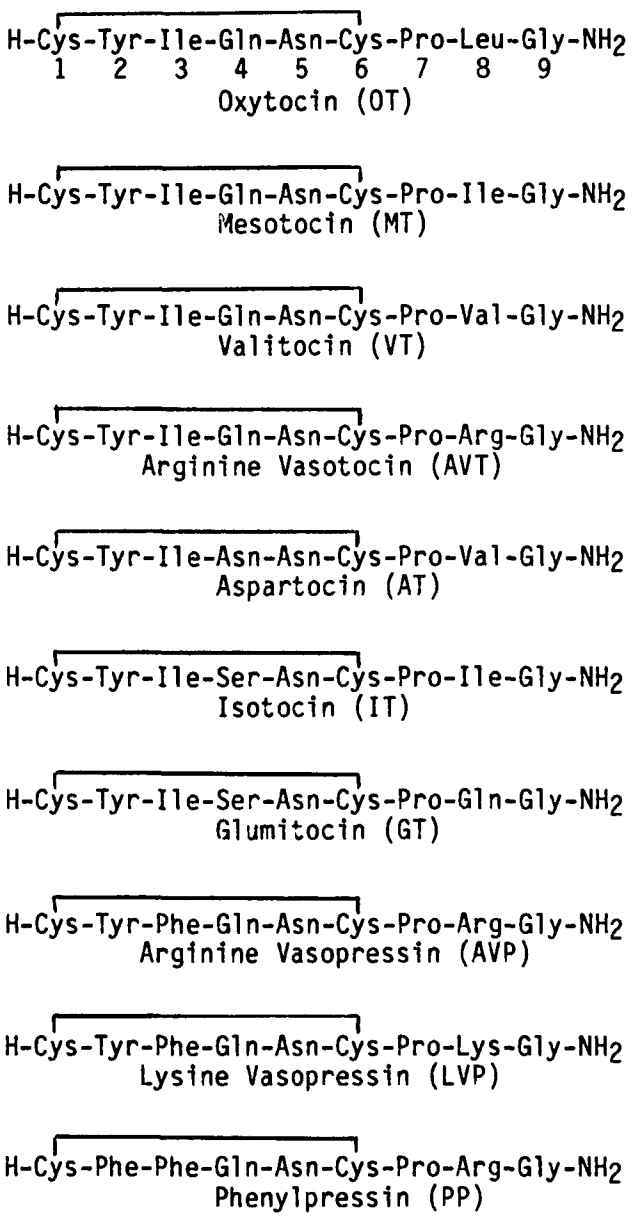


Figure 1. Structures and Shortened Notation for Neurohypophyseal Peptides.

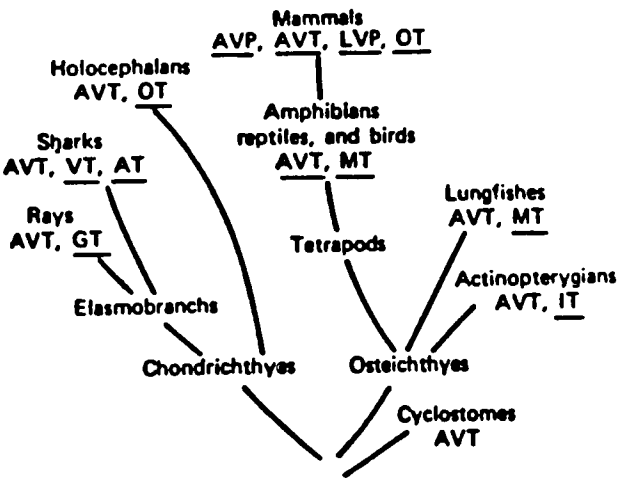


Figure 2. Distribution of Neurohypophyseal Principles Among the Vertebrates.

Table 1. Biological Activities of Neurohypophyseal Hormones.

Compound	Rat Uterus	Avian Vasodepressor	Milk Ejecting	Antidiuretic	Pressor
Oxytocin	546	507	410	2.7	3.1
Mesotocin	289	498	328	1.1	6.3
Valitocin	199	278	308	0.8	9.
Arginine Vasotocin	127	493	210	231	160
Aspartocin	158	201	382	0.17	0.12
Isotocin	145	382	375	0.75	0.05
Glumitocin	10	-	53	0.41	0.35
Arginine Vasopressin	12.	100	30-120	503.	487.
Lysine Vasopressin	4.8	48	31.	203.	243.
Phenylpressin	0.2	< 1.	3.	350.	122.

structural change which appears to account for the biological activity differences is the difference at position 8. If the analogue has a lipophilic residue at this position (Leu, Ile, Val, Gln), oxytocin-like activities are noted, while if the analogue has a basic residue (Arg, Lys) at the 8 position, vasopressin-like activities are obtained (Table 2). It is generally thought that the degree of basicity of the amino acid residue in position 8 of the neurohypophyseal hormones is important for optimizing the pressor or vasoconstrictor properties of these molecules. The length of the side chain in this position may also be of importance while basicity in this position might be very important for affinity of the hormone for its receptor in mammalian vascular muscle, an optimum side-chain length in this position may be critical in determining maximal biological activity.

The human posterior pituitary has three classes of secretory products of which two are neurohormones. These are vasopressin (or antidiuretic hormone; VP) and oxytocin (OT); the third secretory product of the posterior pituitary is made up of the carrier proteins of the neurohormones, the neurophysins. To date these neurophysins have no known biological activity, but they remain of great interest for their close association with the neurohypophyseal hormones as binding proteins both before and after neurosecretion. The neurophysins and the peptides are found loosely bound together in the neurosecretory granules (Dean et al., 1968; Ginsburg and Ireland, 1966) and are usually secreted together (Husain et al., 1975; Legros et al., 1977) by exocytosis. Once secreted into the circulation, the neurophysin-hormone complex dissociates since

Table 2. Naturally Occurring Neurohypophyseal Peptides.

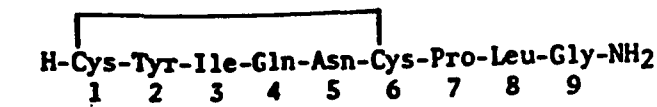
Common structure: Cys-Tyr- - Asn-Cys-Pro- -Gly(NH_2)
1 2 3 4 5 6 7 8 9

Nonapeptides	Amino acid in position 3	4	8	Vertebrates where present
Vasopressor peptides:				
Arginine-vasotocin (AVT)	Ile	Gln	Arg	All vertebrates expect some Suina
Arginine-vasopressin (AVP)	Phe	Gln	Arg	All mammals except some Suina
Lysine-vasotocin (LVT)	Ile	Gln	Lys	Suina
Lysine-vasopressin (LVP)	Phe	Gln	Lys	Suina
Oxytocin-like peptides:				
Oxytocin (OT)	Ile	Gln	Leu	Mammals, holocephali (chimeras, birds, reptiles, amphibians) [disputed]
Mesotocin (MT)	Ile	Gln	Ile	Sarcopterygii (lungfishes), amphibians, reptiles, birds
Isotocin (IT)	Ile	Ser	Ile	Actinoptergii (bony fishes)
Glumitocin (GT)	Ile	Ser	Glu	Some Elasmobranchii (skates)
Asparatonin (AT)	Ile	Asn	Leu	Some Elasmobranchii (sharks)
Valitocin (VT)	Ile	Gln	Val	Some Elasmobranchii (sharks)

the affinity constant of the neurophysins is too low for them to function as binding proteins at circulating concentrations of the hormones.

Properties of Oxytocin

The subject of this thesis will be one of the oxytocin-like peptides found in mammals called oxytocin (Figure 3). Oxytocin possess a 20-membered disulfide ring and an acyclic tripeptide at the C-terminal of the peptide. The acyclic tripeptide H-Pro-Leu-Gly-NH_2 has no activity in the assay system under discussion. In addition, the biological activity of the ring-opened reduced (or sulfhydryl) form of oxytocin, i.e. H-Cys(SH)-Tyr-Ile-Gln-Asn-Cys(SH)-Pro-Leu-GlyNH_2 (Yamashiro et al., 1966) is low and the onset of activity is slow suggesting that in part this activity may reflect oxidation back to the cyclic disulfide. Interestingly, [Ser^1, Ser^6]OT which is pseudoisosteric to the bis-sulfhydryl form is a weak mixed agonist-antagonist at the uterine receptor (Table 3). However, the 20-membered ring moiety tocinamide, H-Cys-Tyr-Ile-Gln-Asn-Cys-NH_2 is a weakly potent agonist in rat uterus assay (Table 3). It appears that though important elements of receptor recognition required for binding are found in the C-terminal tripeptide of oxytocin, all of the transduction message for oxytocin (at least at the uterine receptor) is found in the cyclic 20-membered ring moiety of the hormone. Such findings also demonstrate the importance of the ring for the biologically active conformation of the molecule. Oxytocin occupies a unique position in peptide-hormone research since it was the first peptide hormone whose primary structure was determined (du



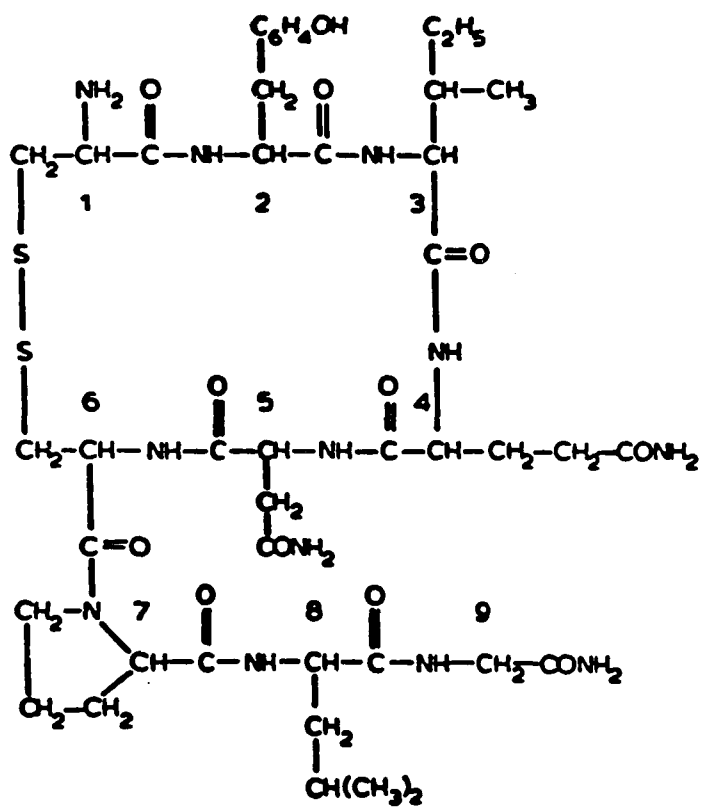


Figure 3. Structure of Oxytocin.

Table 3. Biological Activities of Oxytocin and Selected Analogues.

Analogue	pA_2 Uterotonic (Rat)	Oxytocic Activity (USP) (units/mg)
1. Oxytocin		546
2. Tocinamide		3.2
3. H-Pro-Leu-Gly-NH_2		-
4. [Cys(SH)^1, Cys(SH^6)]-OT		-
5. [Ser^1, Ser^6]-OT		.0012
6. [Pen^1]-OT	6.86	
7. [β-Mpa^1]-(Deamino-oxytocin)-OT		803
8. [dPen^1]-OT	6.94	
9. [γ-Mba^1, HCys^6]-OT	6.74	
10. [N$^\alpha$-Gly]-OT	-	
11. [β,β-(ET)$_2$-β-Mpa^1]-OT	7.24	
12. [β,β-$(CH_2)_5$-β-Mpa^1]-OT	7.43	
13. β,β-(Et)$_2$-β-Mpa^1, Tyr(OMe)^2,Orn^8]-OT	8.91	
14. [dPen^1, Tyr(OMe)^2]-OT	7.76	
15. [dPen^1, Phe^2]-OT	7.78	
16. [Pen^1, Leu^2]-OT	7.14	
17. [Leu^2]-OT		0.45
18. [Tyr(OMe)^2]-OT	6.79	
19. [Thr^4]-OT		900

Table 3 -- Continued

Analogue	pA_2 Uterotonic (Rat)	Oxytocic Activity (USP) (units/mg)
20. [D-Tyr^2]-OT		8.4
21. [Pen^6]-OT		
22. [$\Delta^{3,4}$-Pro^7]-OT		1071
23. [Thi^7]-OT		1180
24. [Orn^8]-OT		42
25. [Arg^8]-OT		127
26. [dPen^1, Orn^8]-OT	7.89	
27. [dPen^1, Arg^8]-OT	6.93	
28. [des-Glycinamide]-OT		15

Vigneaud et al., 1953a) and then proven by total synthesis (du Vigneaud et al., 1953b). Oxytocin, a relatively low molecular weight pituitary peptide (MW = 1007) has a variety of potent physiological effects. Two presently accepted roles of OT in mammals are:

1. Stimulation of female mammary gland to eject milk.
2. Stimulation of smooth muscle contraction in the uterus.

The two functions are undoubtedly of great importance for survival, since they are essential for the young to obtain milk and for the reduction of the period of anoxia of delivery. However, the fact that oxytocin performes a known useful function only a few hours in a lifetime and in only one-half of the species (the female half) has led the investigators to search for other roles for the peptide.

Other functions currently suggested for OT include:

1. It has been shown that oxytocin promotes the uterine prostaglandin release.
2. It has been proposed that OT may assist sperm transport in the female genital tract (Harris, 1947).
3. Oxytocin has been shown to increase ovarian contractility in vitro (Sterin-Borda et al., 1976) and in intraovarian pressure in vivo (Roca et al., 1977) in the rat with a greater response on the day of proestrus in both cases.
4. It has been suggested that OT plays a role in follicular rupture, but this needs further confirmation.
5. In male mammals, oxytocin has been reported to increase semen volume and sperm concentration and/or number per ejaculate

(Kihlström and Melin, 1963; Knight and Lindsay, 1970) as well as sexual drive (Fjellstrom et al., 1968). Oxytocin also stimulates contractions of the male genital ducts and semiferous tubules (Hib, 1974). One study has shown that the inhibition of OT adversely affects fertility.

6. It has been suggested that oxytocin might act as a releasing factor for prolactin. This hypothesis has been infirmed and indeed supra physiologically doses of OT inhibit the prolactin response to suckling in the rat (Kühn et al., 1973). At the renal level, OT has been found to be a diuretic, antidiuretic or natriuretic, according to dosage and experimental conditions (Chard, 1971).
7. Recently, it has been suggested that OT with VP and the small peptides may play a physiological role in memory processes and behavioral control (De Kloet and DeWied, 1980).

The focus of this thesis will be on the role of OT in stimulating contraction of uterus smooth muscle in mammals, particularly humans.

Oxytocin circulates at very low concentrations, and indeed no method developed to date is capable of measuring basal unstimulated levels. The bioassays of OT are usually based on response of the uterus or the mammary gland of small mammals, usually rat or the guinea pig. The uterine assay (Fitzpatrick and Bentley, 1968) can reach a mimimum sensitivity of approximately 10 μU (20 pg) for the perfused everted organ method, and is usually performed for its relative convenience and reliability. The assays based on the response of the mammary gland in

vivo (measuring milk ejection pressure) are generally considered more specific for OT, but less sensitive. Using mammary gland strips in vitro a very sensitive (approximately 1 μU (2 pg)), but less specific assay can be obtained (Fitzpatrick, 1973). The bioassays done for the analogues presented in this thesis includes only in vitro and in vivo uterus assays in rat.

Efforts to understand the chemical physical basis for peptide hormone action have been of limited success owing to two different, but interrelated problems. The first concerns the molecular dynamics of the peptide hormone in solution. Generally, these peptides are small flexible molecules capable of existing in multiple conformational states. The statistical distribution of these conformations are highly dependent on the local environment, which can affect both intramolecular as well as intermolecular interactions. The second problem arises from consideration of the events occurring at the receptor.
Biological information transfer for peptide hormones and neurotransmitters involves two separate, but related events at the receptor: a) recognition (binding) and b) transduction (activation of the biological response) (Rossum, 1962; Hruby, 1981). A difficulty encountered is in defining the conformational and dynamic properties of the peptide that are important for each of these events and how the act of binding is translated into a biological response. One approach to overcome this difficulty and to begin a rationale approach to the design of hormone analogues with specific biological properties is to synthesize analogues that have been conformationally restricted (Hruby

et al., 1982). The basic hypothesis behind such studies is that appropriate conformational constraints will restrict a residue or group of residues to a sufficiently small region of conformational space that when the peptide interacts with its receptor the conformation seen in solution will remain due to the high activation energy or the free energy needed to change that conformation once bound to the receptor. Some of the approaches taken are shown in Table 4. These restrictions utilize changes which are meant to have their primary effect on the backbone conformation. This is consisent with the fact that conformational studies of peptides by biophysical methods have concentrated on defining the backbone ϕ, ψ, and ω angles (Kendrew, 1970), (Figure 4). However, from the standpoint of peptide hormone receptors the overwhelming evidence suggests that the side chain groups on the peptide provide the major loci for recognition (binding) and transduction (biological activity). From this point of view it is the 3-dimensional relationships of the side chain groups in the peptide to one another (topology) that determines the binding message and biological message for a peptide hormone (Hruby, 1981; Hruby et al., 1982). The backbone serves as structural matrix for the side chain groups.

In this paper attention has been given to fixing peptide conformation and topology via side group to side group covalent interaction. Conformationally restricted analogues can provide considerable insight into those conformational and topological features of a peptide that are important for recognition and/or transduction.

Table 4. Some Conformational Restrictions and Structural Features Apparently Related to Antagonist Activity in Oxytocin Analogues

Conformational or Structural Change	Conformational and Structural Effects	Examples of Oxytocin Antagonists
1. β,β-dimethyl (or larger alkyl in half-Cys-1 position)	a) S-S dihedral angle >110°C	[Pen^1]OT
	b) one (or two) C_7 turns	[Pen^1, Leu^2]OT
	c) tripeptide side chain restricted	[Pen^1, Thr^4]OT
	d) Tyr-2 side chain restricted	[Pen^1]OT
	e) Asn-5 side chain restricted	[Pen^1, Leu^2]OT
2. Pen-1 + Leu-2	a) Leu-2 side chain pseudoaxial	[Pen^1, Leu^2]OT
3. D-Amino acid - position 2	a) Stereostructural relation of side chain changed	[D-Tyr^2]OT
4. D-p-Me(Et)-Phe-2 or D-p-Me(EtO)-Tyr-2	a) Side chain more bulky	[β-MPA^1, $Phe(Et)^2$]OT [Pen^1, $Phe(Et)^2$]OT
5. Pen-1 + Thr-4	a) Side chain in position 4 conformational restricted	[Pen^1, Thr^4]OT
	b) Asn^5 side chain restricted	[Pen^1, Phe^4, Thr^4]OT
6. $\Delta^{3,4}$-Pro^7	a) Side chain restricted, more π character	[Pen^1, $\Delta^{3,4}$-Pro^7]OT
7. Orn^8	a) Neutral to basic amino acid	[β,β-$(Et)_2$-β-MPA^1, Orn^8]OT

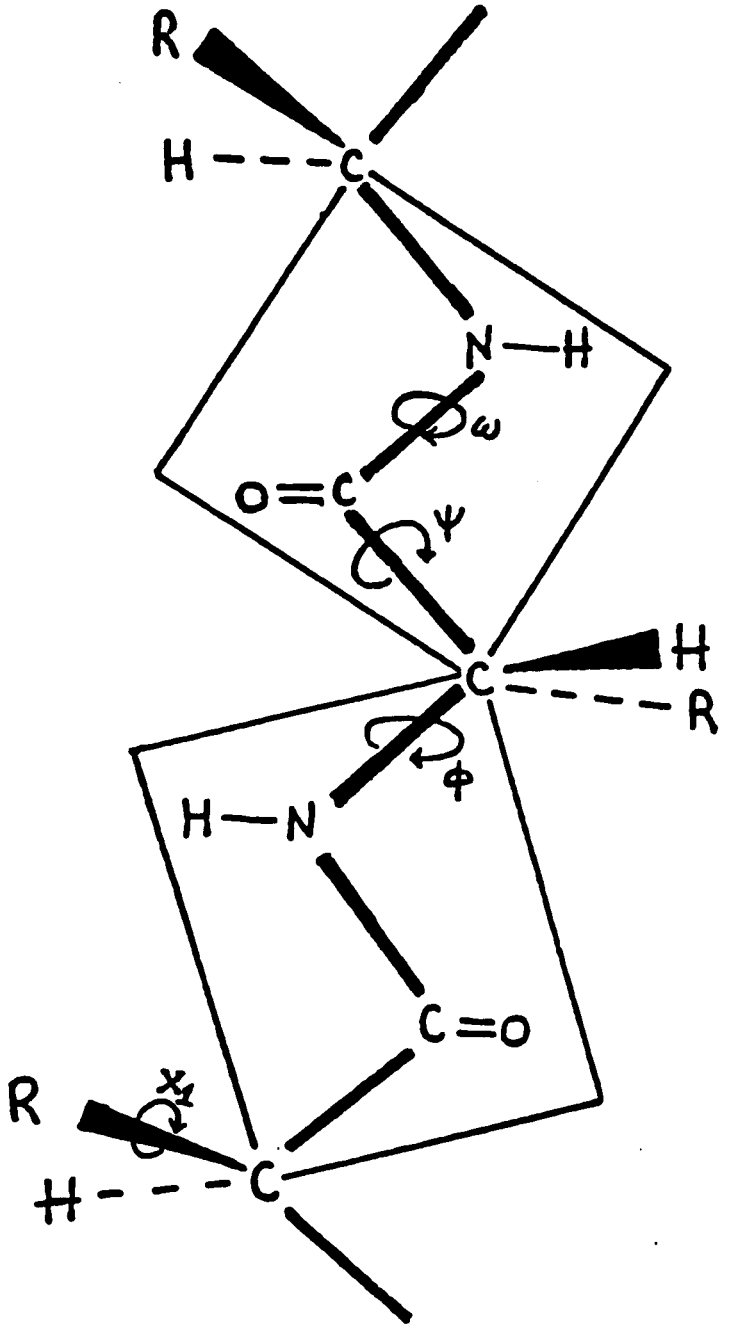


Figure 4. Pictorial Representation of the Peptide Backbone Torsional Angles.

Such modifications may provide agonists with dramatically increased potency; analogues that distinguish the binding from the transduction message, and hence acts as antagonists; derivatives with prolonged activity *in vivo*, analogues with high specificity for a particular receptor; and compounds with resistance to enzymatic degradation in the gastrointestinal tract and hence oral activity.

The development of systemic approaches to the design of peptide hormone antagonists (inhibitor) based on chemical-physical principles has been slow. Rather most of the limited success which has been obtained in this area has resulted from classical structure-activity studies in which amino acid residues are replaced by other residues in the primary sequence to change specific electronic, steric, or other functional characteristics of side chain groups. Possible leads for the design of peptide hormone antagonists usually have come from analogues which were shown to be partial agonists or mixed agonists-antagonists. Then the structural features believed to be important to inhibitor activity were retained, and efforts were made to obtain high antagonist potency by modifications of primary structure in parts of the molecule which were believed to be important for binding. Though some progress has been made by this approach, antagonists which have useful *in vivo* activities are still not available for most peptide hormones.

Oxytocin Antagonists

Perhaps the greatest impetus to the design of potent antagonists came from the reports of du Vigneaud and co-workers (Chan et al., 1967; Schulz and du Vigneaud, 1967) that [Pen^1]OT (Chan et al., 1967) and

the corresponding deamino analogue [dPen^1]OT (Chan et al., 1967) were potent oxytocin antagonists. This led du Vigneaud and associates to introduce the β,β-diethyl (Vavrek et al., 1972) and β,β-cyclopentamethylene (Nestor et al., 1975) groups into the half-Cys^1 position of hormone as their β-mercaptopropionic acid derivatives.
These derivatives were found to be even more potent antagonists than the deamino-penicillamine analogues in the uterotonic assay.
Subsequent studies particularly in the laboratories of Manning and Sawyer, and of Hruby and Chan, have utilized these findings and have made further modifications in positions 2, 4, and 8 of oxytocin to produce even more potent antagonists. Indeed, Manning and Sawyer have reported an analogue which is 50-100 times more potent an antagonist than the early inhibitors, [β,β-Et$_2$-β-Mpa^1,OMe-Tyr^2,Orn^8]OT (Bankowski et al., 1980).

Solution Conformation of OT

Urry and Walter have proposed (Urry and Walter, 1971) a conformation, (Figure 5) as for the most part, a biologically relevant model. A key feature of model is the presence of β-turn involving residue 2-5. This ring conformation is characterized as a cross β-structure involving the sequence -tyrosyl-isoleucyl-glutaminyl-asparaginyl- with the hydrogen bonded ring being closed by the NH of asparagine and the C=O of tyrosine (Walter, 1977). To complete the backbone conformation of oxytocin there is a second β-turn comprised of the sequence -cysteinyl-prolyl-leucyl-glycyl- and utilizing the C=O of the cysteine residue in position 6 and the N-H of the glycine residue to

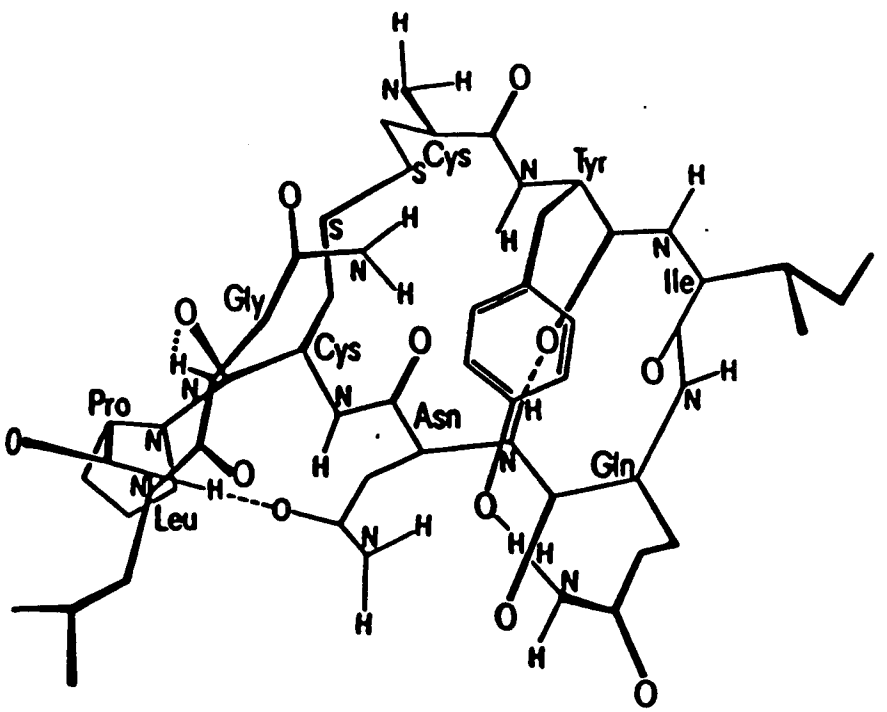


Figure 5. Cooperative Model Proposed by Walter.

close the hydrogen-bonded ring of the β-turn. The second proposed β-turn is further stabilized by hydrogen bonding of the end peptide N-H at leucine to the C=O of the asparagine carboxamide. The tail is free to move in aqueous media (Glasel et al., 1973; Deslauriers et al., 1974), and the tyrosine-2 residue of oxytocin is folded over the 20-membered ring in the proposed biologically active conformer.

Conformation of [Pen^1]OT

The proposed conformation for [Pen^1]OT in aqueous solution (Figure 6), consists of two $C_7(1 \leftarrow 3)$ turns involving the half-Pen^{1}CO and Ile^{3}NH, and the Ile^{3}CO and Asn^{5}NH hydrogen-bond (Mosberg et al., 1981; Meraldi et al., 1975). We have assessed the conformational requirements of the side chain groups of residue 2 and 4 which are at the center of these reverse turns in relation to the biological potency of the antagonists. The 2-position of oxytocin has long been postulated as critical to both agonist (Walter, 1977) and antagonist (Meraldi et al., 1977; Hruby, 1981) activity. In the cooperative model, Walter proposed that placement of the tyrosine aromatic side chain over the 20-membered disulfide ring of oxytocin was critical to agonist activity. This view was supported by the observation that [D-Tyr^2]OT was a partial agonist (Hruby et al., 1979) and that in the antagonist [Pen^1]OT the conformation of the Tyr side chain groups, which would place the aromatic group over the 20-membered ring, was excluded (Hruby, 1981; Meraldi, 1975). Furthermore, the observation that though [Leu^2]OT has only 1/1000 the potency of oxytocin as an agonist [Pen^1, Leu^2]OT is about 3 times more potent than [Pen^1]OT as an antagonist could be

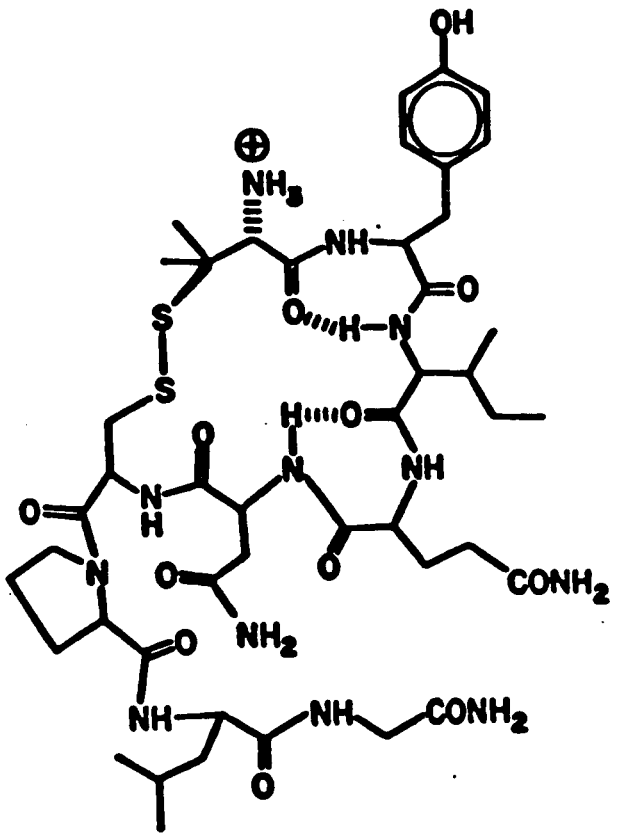


Figure 6. Structure of Pen^{1}-Oxytocin Showing the Proposed Intramolecular Hydrogen Bonding Scheme in its Aqueous Solution Conformation.

explained by the conformational finding that the [Leu^2] side chain group in [Pen^1, Leu^2]OT could assume a pseudo-axial conformation over the 20-membered disulfide ring, but that conformational compensation fixed the Asn^5 and the half-Cys^6 side chain groups in a manner that does not permit transduction. These results provide further evidence for the suggestion that oxytocin antagonists at the uterine receptor utilize different structural and conformational features of their own and of the receptor for interaction with the receptor. The development of an understanding of the conformational and dynamic properties that might be important to the design of oxytocin antagonists began with a report of Hruby and co-workers (Hruby, 1974; Meraldi et al., 1977; Hruby, 1981). In this comparative study conformational properties of oxytocin and the antagonist [Pen^1]OT in aqueous and dimethylsulfoxide solution were examined using primarily nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopies. The major conclusions after comparative studies of the backbone conformation, the disulfide chirality, and the amino acid side chain conformations and dynamic are as follows:

1. Oxytocin has a flexible conformation in aqueous solution; this conformational flexibility is important to agonist activity, facilitating hormone binding to receptor, and obtaining the correct conformation for transduction and for ability to adaption to different receptors during evolution.
2. Oxytocin can exist in several different conformational families depending upon environment.
3. 1-Pencillamine antagonist analogues of oxytocin have different

more rigid backbone conformations than oxytocin in both aqueous and dimethylsulfoxide solution.

4. The side chain conformations of amino acid residues in [Pen^1]OT and [Pen^1, Leu^2]OT are more restricted than in oxytocin, especially at residues 1, 2, 4, 6 and 8.
5. The conformation of [Pen^1]OT contains two (Hruby and Mosberg, 1981) 1 ← 3 reverse turns in aqueous solution. In addition to stabilizing the 20-membered disulfide containing ring backbone conformation of the analogue, this conformation also is consistent with restricted amino acid side chain conformations. A particular interesting result was the exclusion of the tyrosine side chain rotamer which would place the aromatic moiety over the 20-membered disulfide containing ring. It was suggested that this conformational property might be important to the antagonist activity of this analogue.
6. These findings were found to be consisent with certain aspects of the cooperative model of oxytocin agonist activity proposed by Walter (Walter, 1977) and with the dynamic model of oxytocin agonist and antagonist activity by Hruby and co-workers (Hruby, 1981; Meraldi et al., 1977).

In the following section, the importance of each residue in oxytocin to antagonist activity from the perspective of these models will be briefly reviewed. The antagonists will be dealt with exclusively focusing on the binding message only, since an inhibitor cannot transduce the biological message.

Half-Cys^1 Position

Modification of position 1 in oxytocin is critical to development of an oxytocin antagonist at the rat uterotonic receptor. In most cases, increased antagonism results by modifying the 1-position at the α- or β-position with bulky groups. N^{α}-Substitution or side chain lengthening (Table 3; 9, 10) can lead to weak antagonism (du Vigneaud et al., 1960; Schulz and du Vigneaud, 1966). Virtually, all of the most potent antagonists result from disubstitution of the β-hydrogens of half-Cys^1 with alkyl groups (e.g., β,β-dimethyl, β,β-diethyl or β,β-cyclopentamethylene). In general, these substitutions were made with simultaneous replacement of the terminal α-amino group with hydrogen such as in deaminopenicillamine (β,β-dimethyl-β-mercapto-propionic acid). The effect of such substitution on conformation and dynamic properties of the hormone is dramatic as shown by conformational analysis using Nuclear Magnetic Resonance (NMR) (Hruby et al., 1979; 1980), laser Raman and circular dichroism spectroscopies (Hruby et al., 1978). First, the disulfide bridge assumes a conformation in which the C-S-S-C dihedral angle is >100°. Careful examination of CD and Raman results shows that the angle is about 115° and the chirality is right handed (Hruby et al., 1978; Hruby, 1981). Second, the conformations of positions 1 and 6 of the analogue are more restricted, and this, in turn, helps restrict the conformation of the entire analogue.
The restrictions are important to the binding message and antagonist properties of these compounds in at least three ways:

1. Lipophilic (and possibly steric) properties of the β-substituents in the 1-position increase the binding interaction with the receptor.
2. The conformational restriction retards metabolic breakdown.
3. The conformational and dynamic properties of the hormone prevent the hormone-receptor complex from attaining the biologically active conformation. Also conformational changes in the ring moiety of oxytocin are important to the transduction process at the uterine receptor.

Tyr^2 Position

Substitution of tyrosine in oxytocin of deamino-oxytocin by an O-alkylphenol or p-alkylphenyl side chain moiety can lead to analogs with weak to moderate antagonist activities (Table 3; 15-18). Conformational analysis and dynamic studies indicate that when an aromatic residue is in position 2 in Pen^1 analogues, the conformational properties are such that the rotamer which would place the aromatic ring over the 20-membered disulfide ring is excluded and it was suggested (Hruby, 1981; Meraldi et al., 1977) that this is critical to the antagonist activities of these analogues. Interestingly, [Pen^1, Leu^2]OT (Hruby et al., 1979) is a more potent oxytocin antagonist than would be predicted from the agonist activity of [Leu^2]OT (Hruby et al., 1978). Conformational analysis of [Pen^1, Leu^2]OT indicates that in this case the isobutyl side chain group of leucine-2 assumes a pseudo-axial position over the 20-membered ring and is quite flexible, while by conformational compensation the Asn^5 and (half-Cys^6) side chain moiety

becomes conformationally much more rigid. This suggests that the conformational state of the 5-position also is important to agonist and antagonist activity. The high antagonist potencies of the 2-OMe-Tyrosine analogues [β,β-$(Et)_2$-β-Mpa^1,$OMeTyr^2$,Orn^8]OT and [β,β-$(CH_2)_5$-β-Mpa^1,$OMeTyr^2$,Orn^8]OT suggest, however, that a lipophilic aromatic amino acid residue in position 2 can significantly enhance the antagonist potency.

Ile^3 and Gln^4 Positions

These two residues appear to be important primarily to modulate the antagonist potency, though a few 3 or 4 position substitutions per se can provide very weak antagonists (Ray et al., 1975; Smith et al., 1977) presumably by imposing conformation perturbation at position 1 and/or 2 and/or 5 of the hormone. Impressive increases in antagonist potency are seen by replacing the Gln^4 with a Thr^4 residue (Table 3; 19). Conformational analysis indicates that this may be due to the more fixed conformation assumed by the threonine side chain moiety in these analogues.

Asn^5 Position

No studies have been made of oxytocin inhibitors modified at position 5, though this appears to be a critical position for agonist activity (Walter, 1977). Conformational studies on [Pen^1, Leu^2]OT suggest that certain conformational restrictions at this position may be important to antagonist potency. It would be interesting to prepare 5 position analogues with ethyl, 1-propyl or 1-butyl and related side chains to examine the importance of carboxamide group to the oxytocin

binding message and to determine the steric requirements of the 5 position.

Half-Cys^6 Position

Since the early studies which showed [Pen^6]OT to be a weak antagonist (Shulz and du Vigneaud, 1967), very little has been done to examine changes at this position which might induce or enhance antagonist activity in oxytocin analogues. Though conformational analysis results suggest that restricting the conformation at this position might induce antagonist activity. It, therefore, would be interesting to substitute penicillamine or other half-cysteine derivatives with more bulky β-substituents into position 6 in conjunction with the Pen^1 or the O-Me-Tyr^2 substitution.

Pro^7 Position

No substitutions at the 7 position of oxytocin antagonists have been made. This is rather strange, since the 7 position is believed to be important for the binding message (Walter et al., 1976) and both [7-(thiazolidine-4-carboxylic acid]OT, ([Thi^7]OT (Rosamund and Ferger, 1976) and [$\Delta^{3,4}$-Pro^7]OT (Moore et al., 1977; Smith et al., 1977) are more potent agonists than the native hormone. Similar substitutions in potent oxytocin antagonists should make them even more potent.

Leu^8 Position

Conformational studies indicate that in antagonist analogues of oxytocin containing the Pen^1 substitution the 8-position becomes more restricted. However, little has been done to examine the effect of

other lipophilic amino acid residues, especially conformationally restricted ones in this position. A very interesting lead has been obtained in recent studies in which substitution in the 8-position with the basic amino acid residues arginine or ornithine (Table 3; 26, 27); (Sawyer et al., 1980) produced analogues that are more potent antagonists than the 8-leucine analogues. This is particularly interesting since in oxytocin agonist replacement of the 8-leucine residue with a basic residue (Table 3; 24,25) such as arginine leads to analogues that are less potent. This may suggest that oxytocin antagonists have a different binding mode than agonists, at least with regard to the 8-position.

Gly-NH_2^9 Position

[des-Glycinamide^9]OT (Table 3; 28) (Walter et al., 1976) has slight antagonist activity in the avian vasodepressor system, but is a weak agonist at the uterus. Little else has been done to investigate the effects of structural or conformational change in this position on the antagonist potency.

Advantages of Oxytocin Antagonists

1. The fundamental significance of peptide hormone antagonists lies in their ability to be used as tools for probing regions of the native peptide hormone important for transmitting a biological message.
2. They allow for separation of binding message residue from biological message residue.

3. The development of highly potent oxytocin antagonists is useful in determining of molecular events of hormone-receptor interaction. To correlate the observed biological activity with the spatial arrangement and flexibility of amino acid side chain.
4. Such analogues are useful as medicinal agents.

The object of this investigation is to prepare antagonists of oxytocin peptide hormone. The major emphasis in this thesis will be to examine oxytocin structure-function relationship from the vantage point of receptor recognition (binding, potency), transduction (agonist, partial agonist, and antagonist), and reversal (prolongation).
With that in mind, the focus of this investigation has been the following:

1. Incorporation dehydroamino acids in binding site of oxytocin (position 7 and 8).
2. Restriction of amino acid side chains at positions 1 and 2
3. Design of peptide hormone analogues with enhanced uterotonic response and greater antagonistic potency in vivo which could be used to prevent pre-term labor.

We can differentiate between three types of alteration (Walter et al., 1971 and references therein) when considering structural modifications:

a. Those affecting the stabilization of the backbone of the peptide which would extensively perturb the spatial relationships among the constituent amino acid, and hence affect both binding and intrinsic activity uniformly.

b. Those which while retaining the stability of the backbone conformation alter the steric environment and charge distribution of limited surface areas, and thereby can affect affinity and intrinsic activity differentially.

c. Those changing the steric and electronic requirements of moieties comprising the active surface of the peptides without perturbing the peptide backbone of the hormone molecule, and hence affecting intrinsic activity without altering affinity.

Since the ultimate goal is to design antagonists with prolonged activity, we are interested in those modifications that enhance affinity, but decrease or abolish intrinsic activity. In the cooperative model of oxytocin the chemically active groups (3-carboxamide groups, the phenolic hydroxyl group, and the acyclic propyl-leucyl-glycinamide moiety) are oriented toward the same side of the 20-membered cyclic component of the hormone. Such an arrangement results in a hydrophilic region, which is believed to be important for the expansion of the inherent catalytic activity (i.e. intrinsic activity) of the hormone. Certain structural modifications in this hydrophilic region, particularly if hydrophobic in character might be anticipated to reduce the intrinsic activity of the peptide.

The binding elements in addition to their topological arrangement of being at the corner positions of the two β-turns, which makes them most visible to the receptor, they share a common chemical nature in their lipophilicity. In principle, they can be modified to provide greater affinity with the receptor.

CHAPTER 2

EXPERIMENTAL METHODS

General Synthetic Methods

All optically active amino acids are of the L variety unless otherwise stated. Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical-Nomenclature (Kendrew et al., 1970).

Capillary melting points were determined on a Thomas-Hoover (Arthur H. Thomas Co., Philadelphia, PA) melting point apparatus and are uncorrected. Peptide purity was established by thin layer chromatography (TLC) which was performed in glass-backed Silica gel G-plates (E.M. Science, Gibbstown, NJ) using the following solvent systems:

1. Butanol:Acetic Acid:Water:Pyridine

   6 : 1.2 : 4.8 : 6

2. Butanol:Acetic Acid:Water:Pyridine

   15 : 3 : 10 : 12

3. Amyl Alcohol:Pyridine:Water

   7 : 7 : 6

Detection was made by iodine vapor and ninhydrin. In all cases single symmetrical spot was observed for purified materials.

High performance liquid chromatography (HPLC) was performed on a Spectra Physics Model 8700 (Spectra Physics Corporation, San Jose, CA)

instrument equipped with a Spectra Physics Model 8400 variable wavelength detector. Analytical samples were detected at 214 nm and load size per run was 25-50 µl using Vydac C-18 column (#830822, 25 x 0.46 cm, id). Final high performance liquid chromatography purification of peptide samples were performed according to the protocol developed by Blevins, Burke, and Hruby (1980), but using a Vydac C-18 reverse phase column (Separation Group, Inc., Hesperia, CA). Semi-preparative HPLC purifications were performed on a Waters C_{18} reverse phase column 10 cm long x 0.8 cm id (Waters Associates, Milford, MA) contained in a Radial Compression Module (RCM). The mobile phase for each column consisted of a starting mixture of (90%) 0.1% TFA (trifluoroacetic acid) and (10%) CH_3CN (acetonitrile). The peptides were purified by a gradient running from 10% CH_3CN → 50% CH_3CN in 30 min at a flow rate of 5.0 ml/min or 1.5 ml/min for preparative HPLC or analytical HPLC, respectively. The purification of peptides were monitored at 280 or 214 nm. HPLC samples for purification were prepared by dissolving a preweighed peptide sample of 80-100 mg in the beginning elution mixture of solvents (90% CH_3CN:10% TFA) 1-2.0 ml. TFA solutions were prepared by adding the appropriate amount of TFA 1 ml to 999 ml HPLC pure H_2O. Purified by filtration of deionized H_2O through HPLC, a norganic carthrige (Millipore Corp., Bedford, MA). Before use, the resulting solution was filtered through a Millipore filter (HAWP, 0.45 µm) and degassed for 30 min-60 min. The acetonitrile was also filtered through a Millipore filter (RNLP, 0.5 µm) and degassed prior to use. The HPLC chromatograms indicated purities in excess of 98%.

Optical rotation values were measured using a Rudolph Research (Flanders, NJ) Autopol III polarimeter. The samples were prepared by dissolving 5-10 mg (measured accurately) of purified peptide in 2 ml 10% HOAc, in case of amino acids 15-30 mg was dissolved in 1.0 ml 1.0 N HCl. Amino acid analyses were obtained by the methods of Spackman, Stein, and Moore (1958) on a Beckman Instruments, Inc. (Fullerton, CA) 120C amino acid analyzer following hydrolysis. Hydrolysis involving purified peptide was done in methanesulfonic acid (MSA) containing 0.2% 3-(2-aminoethyl)-indole or 3 M mercaptoethane sulfonic acid at 110°C for 22-40 hrs. Following the hydrolysis the sample was diluted with 3.5 N NaOH to adjust the pH between 1-2. No corrections were made for destruction of amino acids during hydrolysis.

N^{α}-Boc amino acids and amino acid derivatives were purchased from Vega Biotechnologies, Inc. (Tucson, Arizona), Chemical Dynamics Corporation (South Plainfield, New Jersey), and Bachem (Torrence, California), or were prepared using published procedures. All amino acid derivatives were tested for homogeneity by TLC in solvent systems 1, 2, 3. TLC was performed on Baker Flex Silica gel 1B-F plates (J. T. Baker Chemical Co., Pittsburgh, NJ) in three solvent systems.

1. Chloroform/Methanol (95:5)
2. Chloroform/Methanol (65:35)
3. Acetone/Acetic Acid (98:2)

Detection was made with UV, Iodine, and Ninhydrin. The purity of amino acid were also checked by NMR, melting point, and Ninhydrin Test. ^{1}H Nuclear Magnetic Resonance (NMR) spectra were obtained using a Bruker

WM 250 FT NMR spectrometer (Bruker Instruments, Inc., Billerica, MA) equipped with an Aspect 2000 A Computer, Varian T-60 NMR Spectrometer (Varian Associates, Inc., Palo Alto, CA). Peptide samples for NMR spectra measurements were prepared according to methods previously reported by Mosberg (Mosberg et al., 1982). Approximately 5-7 mg of the purified peptide was dissolved in 1-2.0 ml D_2O containing 8 µl acetic acid-d_4, to exchange water labile protons with deuterium and lyophilized. H-1 NMR samples were then prepared by dissolving this lyophilized powder in 99.96% D_2O (Aldrich Chemical Co., Milwaukee, WI) plus 3-4 drops of acetic acid-d_4 as reference. ^{13}C NMR were obtained on Bruker AM-250, peptide samples for C-13 NMR were prepared by dissolving 20-40 mg of purified peptide in 2 ml 99.96% D_2O and 3-4 drops acetic acid-d_4. IR spectra were obtained using a Perkin Elmer Model 630 scanning spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Ultraviolet spectroscopic measurements were performed on a Perkin Elmer Model 440 scanning UV-VIS spectrometer.

General Procedure for Synthesis of N^{α}-Boc Amino Acids

All N^{α}-Boc amino acids were synthesized by the literature procedure of Turbell (Turbell et al., 1972). To a cold (0°C) solution of 10 mmoles of amino acid in 30 ml dioxane/H_2O (2:1) was added 10 ml of 1.0 N NaOH. Then 11 mmoles of Boc-dicarbonate was added and the mixture was stirred overnight at room temperature. The dioxane/H_2O was removed under vacuum. When all the solvent was removed, 20 ml of H_2O and 15 ml of ethyl acetate was added back and cooled in an ice-bath. The pH was adjusted to approximately 2.5 with cold 1.0 N HCl. All the following

extractions were done with cold solvents. The aqueous layer was extracted with (5 x 15 ml) ethyl acetate. The ethyl acetate fractions were pooled and extracted with 2 x 20 ml saturated aqueous NaCl solution and 2 x 20 ml H_2O. The ethyl acetate layer was dried over anhydrous $MgSO_4$ at room temperature. The solution was filtered and dried in vacuo. Recrystallization was done with ethyl acetate/hexane or petroleum ether and crystals resulted in all cases except for N^{α}-Boc-(S-4-methylbenzyl)penicillamine which was an oil. Yields range from 50-90% depending on the amino acid.

Preparation of Amino Acid Derivatives

Synthesis of S-4-Methylbenzylcysteine

Following the reported procedure by Erickson and Merrifield (Erickson et al., 1973), triethylamine (55.5 mmol, 7.75 ml) and 4-methylbenzylchloride (18.5 mmol, 1.7 ml) were added to a solution of cysteine hydrochloride monohydrate (18.5 mmol, 3.25 g) in 2:1 (v/v) ethanol/H_2O (30 ml). The mixture was stirred for 12 hours and filtered. The filtered cake was washed well with H_2O and recrystallized from 3:2 (v/v) ethanol/H_2O to give product; white solid; m.p. = 209-211°dec; yield 93% . The TLC plates were developed with 17:2:1 (v/v/v) chloroform-methanol-acetic acid (CMA) or 7:2:1 (v/v/v) benzene-methanol-acetic acid (BMA). The plates were visualized by spraying with 0.2% ninhydrin in 1-butanol and heating. R_f (CMA) = 0.19; R_f (BMA) = 0.42.

NMR (TFA) 2.37 (s 3H, CH_3)

3.1-3.3 (m 2H, CH_2)

3.84 (s 2H, CH_2Ar)

4.1-4.3 (m, 1H, $C^{\alpha}H$)

7-8 (S 4H, Ar)

Synthesis of S-4-Methylbenzylpenicillamine

S-4-Methylbenzylpenicillamine was prepared according to a procedure described by du Vigneaud (du Vigneaud et al., 1930), with some modifications. The penicillamine (42 mmole) was dissolved in approximately 200 ml of anhydrous NH_3 (freshly distilled from Na). The solution was treated with Na (42 mmole) that was washed with absolute diethyl ether and weighed under paraffin oil. After the blue color persisted for 10 minutes, α-chloro-p-xylene (46 mmoles) was added dropwise via the pressure equalizing addition funnel over a 30 minute period once the mixture began a gentle reflux. After the α-chloro-p-xylene was added, the NH_3 was removed with a nitrogen stream. Once all the NH_3 has been removed 80 ml of H_2O was added and the solution was transferred to a separatory funnel. The unreacted halide was then extracted with absolute diethyl ether (2 x 40 ml) which was discarded. The aqueous solution was cooled in an ice bath and the pH was reduced to 5.3 with glacial acetic acid. The precipitate was filtered and dried under vacuum. The product was recrystallized from hot H_2O or 20% MeOH; white solid, m.p. = 202-203°C; yield 97%; TLC R_f (CM 95:5) = 0.23, R_f (CM 65:35) = 0.36; R_f (acetone/acetic acid 98:2) = 0.70. NMR (EtOAc):

1.0 - 2.0 (s, 6H, $-C-(Me)_2$)

2 - 3.0 (s, 3H, $-CH_3$)

3.6 - 3.8 (s, 2H, -S-CH_2)

7 - 8.0 (d, 4H, Ar)

Tyrosine Methyl Ether Hydrochloride

Anhydrous methanol (300 ml) was cooled to -30°C in a dry-ice acetone bath, whereupon 39.3 g (330 mmole, 25 ml) of thionyl chloride was added dropwise over a period of 20 min. After addition of the thionyl chloride, the mixture was stirred for 5 min in the cold. Then, 50 g (280 mmole) L-tyrosine was added to the cold mixture over a period of approximately 5 min yielding a white slurry. The slurry was stirred first in the cold for 1 hr, and then for 2 days at room temperature. During the course of stirring at room temperature, the white slurry became a homogeneous mixture. The mixture was evaporated to dryness in vacuo leaving a white solid. The solid was redissolved in methanol (2 x 150 ml) and evaporated to dryness (twice) to further dry the solid. The copious white solid was dissolved in 350 ml dry methanol and then triturated with anhydrous ethyl ether until the solution became turbid. The turbid solution was stirred in an ice-bath, whereupon a flocculent white precipitate formed. The solution was filtered and then dried overnight in vacuo; M.P. = 189-190°C; NMR (TFA); 6.98 (q, 4H, J = 8.0 Hz); 4.6 (dd, 1H); 3.6 (S,3H); 2.3 (d,2H;J = 6.5 Hz); TLC : R_f = 0.12 [acetone-acetic acid (98:2)].

Tyrosine Methyl Ether

The hydrochloride salt of tyrosine methyl ether (10 g, 43 mmoles) was added to 40 ml of an aqueous solution containing 5.9 g (43 mmoles)

potassium carbonate. The resulting mixture was stirred for 10 min at room temperature whereupon a copious white precipitate formed. The precipitate (neutralized tyrosine methyl ether) was filtered and used immediately in the next step of the procedure.

N^{α}-Boc-Tyrosine Methyl Ether

The neutralized tyrosine methyl ether was added to a mixture of a 100 ml tert-butyl alcohol and 150 ml anhydrous ethyl ether, and the remaining slurry stirred at room temperature whereupon 9.38 g (43 mmoles) Boc-dicarbonate was added dropwise to the mixture over a period of 5 min. After addition of Boc-dicarbonate, the stirring mixture began to turn clear. Stirring was then continued for 2 more hrs. After the stirring period, the mixture was further diluted with 150-ml ethyl ether. The resulting solution was extracted with water (3 x 100 ml), and the organic phase was dried over anhydrous magnesium sulfate. The solution was filtered, evaporated to dryness, yielding a clear, oil residue. The residue was dissolved in 50 ml toluene and the resulting solution was stirred in an ice-water bath for 4 hrs to induce crystallization. The crystals that formed were filtered, then dried overnight in vacuo; M.P. = 101-103°C.

NMR ($CDCl_3$): 6.98 (q, 4H; J = 8.9 Hz); 4.6 (dd, 1H)
3.6 (s, 3H); 2.3 (d, 2H; J = 6.5 Hz)
1.4 (s, 9H)

Immobilization of Aminoacyclase on DEAE Sephadex A-25

100 g DEAE-Sephadex A-25 (3.5 ± 0.5 meq/g, bead type) was suspended in 1 liter distilled H_2O at room temperature (Tosa, 1967). This was then added to 5 liter of 0.1 N NaOH and stirred for 3 hours and filtered. The precipitate was washed with H_2O until washing solution was neutralized. The precipitate was then stirred in 5 liter 0.1 M phosphate buffer for 3 hours and allowed to stand for 16 hours; and filtered. This step was followed by stirring the precipitate with 1.67 liter aminoacylase solution for 3 hours followed by filtration. The suspension was then stirred in 5 liter H_2O for 1 hour and filtered. The precipitate was then stirred in 5 liter of 0.2 M NaOAc for 1.0 hour and filtered. The precipitate was then washed with H_2O until no protein was detected in the wash. To store, the precipitate was suspended in 2.5 liter H_2O and lyophilized: optimum pH = 7.0; optimum temp = 42°C; activation energy = 7.0 Kcal/mole; optimum $[Co^{+2}]$ = 0.5 mM; Km = 8.7 mM; V_{max} = 3.33 µmole/hr; half-life = 65 days (50°C).

Resolution of N-Acetyl-D,L-4,5-Dehydroleucine

N-Acetyl-D,L-Dehydroleucine (5.0 g) was dissolved in 254 ml KH_2PO_4/CH_2Cl_2 buffer. The concentration of the mixture of amino acid in buffer was adjusted to be 0.1-0.2 molar. The pH was adjusted to 7.0 with 4.0 N NaOH and 1.0 gram of aminoacylase resin was added to the mixture. The mixture was allowed to stir gently in a H_2O-bath at 45-50°C for 3 days. Following the reaction period the resin was filtered off, and the pH of the filtrate was adjusted to 2.0 with 1.0 N HCl and the unreacted acetyl derivative (N-acetyl-D-Dehydroleucine) was

extracted with EtOAC (ethyl acetate) (5 x 250 ml). The aqueous layer was concentrated in vacuo to about 1.0-5.0 ml. The aqueous layer containing the L-dehydroleucine was then applied to a Dowex Ion Exchange Column AG-50 (Cl^- form) to remove salts. The resin was first regenerated by the following procedure: (using 10-20 equiv. resin/equiv. of compound): 1. Washed with 1.0 N NaOH (3 void volume); 2. Washed with H_2O until pH = 7.0; 3. Washed with 1.0 N HCl (3 void volume); 4. Washed with H_2O until pH = 7.0; 5. The compound was loaded at this point. Following application of the compound, the column was washed with H_2O (3 void volumes) to elute any remaining N-acetyl-D-amino acid derivative. The column was then washed with 5-20% NH_4OH (3 void volume) to elute the unprotected amino acid (i.e., L-Dehydroamino acid). The NH_3 was removed from NH_4OH washes on the H_2O aspirator and the volume was then reduced in vacuo and lyophilized giving a white-yellow powder.

NMR (D_6DMSO/TFA) 1.4-1.6 (s, 3H, $-CH_3$)
2.2-2.6 (d, 2H, $\overset{\beta}{-CH_2}$)
3.6-4.0 (s, 1H, $\overset{\alpha}{-CH}$) (Broad)
4.6-4.8 (s, 2H, $\overset{\delta}{-CH_2}$)
8.0-8.2 (s, 1H, -NH) (Broad)

$[\alpha]_{589}^{25} = -30.77$; $[\alpha]_{546}^{25} = -35.92$

Preparation of N^{α}-Boc-L-4,5-Dehydroleucine

The general procedure for preparation of Boc amino acids was followed. The product was recrystallized from EtOAC/petroleum ether (yield 78.12%); M.P. = 54-56°C.

Preparation of p-Methylbenzhydrylamine Resin (p-MBHA)

This procedure is modified from the literature (Hruby et al., 1977). To 30.0 gram of polystyrene (Copolymerized-1% divinylbenzene; Biobeads; Bio Rad S-X_1, 200-400 mesh, Bio Rad Laboratories, Richmond, CA) beads in 400 ml dichloromethane (CH_2Cl_2) was added 20 ml of p-toluylchloride. The reaction was cooled to 0°C and kept under nitrogen to exclude any moisture. $AlCl_3$, 13.8 g, was added in 3 portions and the reaction was stirred for two hours at 0°C. The reaction was then allowed to warm to room temperature and stirred for 1.5 hours. The reaction was then brought to reflux for 2.5 hours. The reaction was cooled to room temperature and poured into 400 ml of ice-H_2O, which was then filtered off. The resin was washed successively with 3 x 150 ml each of water, methanol and ethanol. The resin was next added to 450 ml H_2O containing 75 ml of concentrated HCl, stirred for one hour, and then filtered off and washed with water. The resin was then added to 150 ml 0.5 N NaOH, stirred for 10 minutes, filtered off and washed successively with 3 x 150 ml each of water, 50% ethanol, ethanol, CH_2Cl_2 and then dried in vacuo. This gives approximately 45 gram of a cream colored p-methylphenylketone resin. IR(KBr):3100, 1650, 1600 cm^{-1}.

A one liter, three neck round bottom flask containing 400 ml of aqueous ammonia (concentrated, 44%) was cooled to 0°C and 340 ml of formic acid (88%) is slowly added. The solution was then heated and H_2O distilled off until the internal temperature reaches 145°C (about 400 ml H_2O is removed). At this time 20.0 g p-toluyl ketone resin (above) was added and the flask was then equipped with a mechanical stirrer and a

reflux condenser. The mixture was stirred for 48 to 72 hours in an oil bath at 170-172°C. The mixture was then cooled, filtered and washed successively with 4 x 50 ml each of H_2O, methanol, and CH_2Cl_2, and dried in vacuo. The resin was placed in a 500 ml round bottom flask with 80 ml concentrated HCl and 80 ml propionic acid and allowed to stir for 5 hours at 110°C. The mixture was cooled, filtered and washed successively with 3 x 50 ml each of H_2O, ethanol, CH_2Cl_2, 10% diisopropyl ethylamine (DIEA) in CH_2Cl_2, CH_2Cl_2 and then dried in vacuo.
This yields between 17 to 19 gram of the title compound; IR (KBr); 3400, 3050, 1650, 1600 cm^{-1}. Amine substitution levels between 0.15-1.0 mmole/gram resin were determine using the picric acid test (Gisin et al. 1972).

Solid Phase Synthesis of Protected Oxytocin Nonapeptide

Each protected peptide was prepared utilizing solid-phase peptide synthetic methodology on a semiautomated instrument designed and built in the chemistry laboratory at The University of Arizona (Hruby, Barstow, and Linhart, 1972), or in an automated instrument (Model 250, Vega Biochemicals, Tucson, AZ). The oxytocin analogues were synthesized by solid-phase method similar to that described by Upson and Hruby (1976) (dicyclohexylcarbodiimide coupling) or by the symmetrical anhydride coupling method. The general coupling schemes used for the synthesis of protected nonapeptides are shown in Tables 5 and 6.
In cases where the symmetrical anhydride method was used, the Boc-amino acid 2 eqiv/DCC (2:1) were reacted at 0°C for 15 min in CH_2Cl_2.
The precipitate (DCU) was filtered off and the anhydride solution was

Table 5. Solid-Phase Peptide Synthesis Experimental Protocol for Coupling with Dicyclohexylcarbodiimide.

Step	Purpose	Solvent or Reagent	Time (min)	Repetition
1	Wash	CH_2Cl_2	1	4
2	Deprotection	TFA-CH_2Cl_2-Anisole (50:48:2)	2	1
3	Deprotect	TFA-CH_2Cl_2-Anisole (50:48:2)	20	1
4	Wash	CH_2Cl_2	1	3
5	Neutralize	DIEA-CH_2Cl_2 (10:90)	2	2
6	Wash	CH_2Cl_2	1	4
7	Analysis	Ninhydrin Test	-	1
8	Couple	Boc-amino acid (3 eq)/CH_2Cl_2 (3 mls), HOBT (3 equiv)/DMF		1
		DCC (2.4 equiv)/CH_2Cl_2	45-90	1
9	Analysis	Ninhydrin Test	-	1
10	Wash	CH_2Cl_2	1	3
11	Wash	100% EtOH	1	4
12	Wash	CH_2Cl_2	1	
- - - - - - - - - -		-Additional Coupling (if necessary)-		- - - - - - - - - -
13	Couple	Boc-amino acid (1.5 eq)/CH_2Cl_2	-	
		HOBT (1.3 equiv)/DMF		
		DCC (1.2 equiv)/CH_2Cl_2	45-90	
14	Analysis	Ninhydrin Test	-	
15	Wash	CH_2Cl_2	1	3
16	Wash	100% EtOH	1	3

Table 6. Solid-Phase Peptide Synthesis Experimental Protocol for Nitrophenyl Ester Coupling.

Step	Purpose	Solvent or Reagent	Time (min)	Repetition
1	Wash	CH_2Cl_2	1	4
2	Deprotection	TFA/CH_2Cl_2/Anisole (50:48:2)	2	1
3		TFA/CH_2Cl_2/Anisole (50:48:2)	20	1
4	Wash	CH_2Cl_2	1	3
5	Neutralize	DIEA/CH_2Cl_2 (10:90)	2	2
6	Wash	CH_2Cl_2	1	4
7	Wash	DMF	1	3
8	Analysis	Ninhydrin Test	-	1
9	Coupling	Boc-amino acid Nitrophenyl ester (4 eq)/DMF HOBT (4 eq)/DMF	- 240-360	1 1
10	Analysis	Ninhydrin Test	-	1
11	Wash	DMF	1	3
12	Wash	CH_2Cl_2	1	4
13	Wash	100% EtOH	1	3
- - - - - - - - -	-Additional Coupling (if necessary)-			- - - - - - - - -
14	Wash	DMF	1	4
15	Couple	Boc-AA-ONP (2.5 eq/DMF) HOBT (2.0 eq)/DMF	- 120-180	1 1
16	Analysis	Ninhydrin Test	1	1
17	Wash	DMF	1	3
18	Wash	CH_2Cl_2	1	3
19	Wash	100% EtOH	1	3

used immediately. Each coupling step was monitored for completeness using the Kaiser ninhydrin test (Kaiser et al., 1970) or chloranil test (Christensen, 1979). N^{α}-Boc protected amino acids were used throughout and p-methylbenzyl protection was utilized for the penicillamine and cysteine sulfurs. Tyrosine was used without side chain protection. A p-methylbenzhydrylamine (p-MBHA) resin was used as the solid support for the synthesis of all oxytocin analogues (1.1 mmoles/gram). In all cases the ninhydrin test was negative indicating >99.4% coupling. For all peptides the terminal Boc-group removed upon completion of synthesis to protect alkylation of tyrosine side chain. The neutralized resin was removed from the peptide synthesis vessel by washing the resin with N,N-dimethylformide (4 x 30 ml) into a sintered glass funnel followed by CH_2Cl_2 washes (4 x 30 ml) and dried in vacuo overnight and then weighed.

Cleavage of the Peptide from the Solid Support

The oxytocin analogues were cleaved from the solid support by anhydrous HF in the following manner: 1.0 g of dried resin was treated with 10 ml of anhydrous HF and 1 ml anisole at 0°C for 45-60 minutes (Stewart and Young, 1969). This procedure will remove the peptide from the resin as well as removing all of the amino acid side chain protecting groups. After the reaction time was completed, all of the solvents were removed in vacuo and the product was washed with dearated solvents, 3 x 30 ml ethylacetate to remove traces of anisole and discarded. The resin was then extracted with 3 x 30 ml each 30% HOAc,

0.2 N HOAc and H_2O. The aqueous extracts were then lyophilized to a crude powder.

Cyclization of Oxytocin Analogues (Disulfide Formation)

The resulting lyophilized white powder from HF is dissolved in deaerated 0.1% aqueous acetic acid to a concentration of approximately $1.5 - 2.0 \times 10^{-4}$ molar. The pH of the peptide solution was adjusted to 8.5 with 3 N NH_4OH and then oxidized with addition of excess 0.01 N $K_3Fe(CN)_6$ (50-80 ml) to form the disulfide bond. The pH of the solution was rechecked and readjusted to 8.5, if necessary. The peptide solution was stirred for 1.0-1.5 hours until the presistence of yellow color indicative of complete disulfide formation was observed. The peptide solution was then acidified with 20% acetic acid to pH = 4.5.
The 5-10 ml (settled volume) anion exchange resin (Rexyn 2.3 (Cl^- cycle) Fisher Chemical Company, Pittsburgh, PA) or Bio-Rad 3-X4A (Bio Rad Laboratories, Richmond, CA) was added and stirred for 20 minutes to remove excess ferro and ferricyanide ions. The resin was removed by filtration and washed with 20% HOAc (3 x 20 ml) to ensure complete exchange of the peptide material from the resin. Approximately 75.0 ml of 1-butanol (reduce bumping) was added to the aqueous peptide solution and concentrated *in vacuo* at 20-30°C to about 500 ml and lyophilized giving a white yellowish residue.

Purification of Oxytocin Analogues

Solvents for gel chromatography were purified by distillation as previously reported by Hruby and Groginsky (1971). The crude

lyophilized material obtained after oxidation of the sulfhydryl groups (position 1 and 6). Served as starting material for the purification. At this stage the sample contained in addition to the active peptide, inorganic salts and some dimeric peptide. The first step in the purification entailed desalting of the peptide with 50% or 30% acetic acid. In a typical run the procedure used was as follows: the crude lyophilized powder (300-600 mg) was dissolved in 2.0 ml of 50% or 30% acetic acid and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40-120 μ, column size (110 x 1.2 cm) which has been pre-equilibrated with 500ml of 50% acetic acid or 30% HOAc. The sample was then washed into the column with an additional one ml of 50% HOAC and eluted with 50% acetic acid at a rate of 8-10 ml per hour. 2.0 ml aliquots were collected using an automated fraction collector. The eluate was assayed for the peptide by U.V. absorption (λ = 280 nm) for those peptides containing tyrosine. For those where tyrosine was not present in the sequence the peptide was assayed by Folin Lowry method (Lowry et al. 1951). The peptide material was eluted in two partially resolved peaks, clearly separated from the salt (tubes 60-80). The peak corresponding to the active peptide was pooled and lyophilized. The lyophilized fluffy powder was then subjected to a final purification on reverse phase HPLC.

CHAPTER 3

DATA AND DISCUSSION

The comparative studies of oxytocin and [Pen^1]OT clearly indicate that agonists and antagonists have different conformational structure requirements for hormone-receptor interaction. One approach to examine the structure, conformation, and dynamic properties of the hormone is design of antagonists of native hormone, oxytocin.
The fundamental significance of peptide hormone antagonists lies in the ability of these compounds to be used as tools for probing regions of the native peptide hormone important for transmitting a biological message.

A solution conformation was postulated by Hruby (Hruby, 1981) for OT antagonists. The features are:

1. A more rigid well-defined peptide backbone for antagonists in aqueous solution.
2. Presence of 1 and sometimes 2 intramolecular H-bonds (3 ← 1, C-7 turns).
3. Restricted side chain conformations of the Pen^1, Tyr^2, Gln^4, Cys^6 residues.
4. A disulfide dihedral angle at ≈ 115° with right-handed chirality.

Through these conformational modifications it has not only been shown that enhanced receptor interaction of the antagonist provides

increased inhibitor potencies, but also it has been possible to determine the contribution of individual amino acid residues toward biological activity (Table 7), and to propose relationships of conformation of oxytocin to biological activity at uterine receptor (Table 8). Design of inhibitors from the standpoint of conformational properties is based on two considerations: 1. Reducing the flexibility of the hormone of particular amino acid residue in a manner compatible with binding to the receptor. The reduced flexibility involves restriction of either the backbone conformation or the individual amino acid side chains or both. The restriction of side chain conformation of individual amino acid residues in peptide hormones is an alternative to peptide backbone conformational restriction. Such restrictions can be achieved by:

1. Introduction of double bond between C_α, C_β, and C_γ bonds of amino acid side chain to enhance π character and to fix the dihedral angle X_1.
2. Introduction of bulky or sterically crowded side chain residue at C-β or other carbon atoms.

The restrictions limit the possible conformations available to peptide analogue.

A comparison of the primary structure of oxytocin and the other eight characterized neurohypophyseal nonapeptides found in nature reveals that mutations during evolution have occurred only at position 3, 4 and 8 (Acher, 1974). The conformation of oxytocin proposed by Urry and Walter places these residues and the residue in position 7 in all

Table 7. Conformation-Activity Analysis of Oxytocin. Contribution of Individual Amino Acid Residues Toward Biological Activity.

Binding Element	
1. isoleucine	Residue 3
2. Glutamine	Residue 4
3. Proline	Residue 7
4. Leucine	Residue 8
Biological Message Element	
1. Tyrosine	Residue 2
2. Asparagine	Residue 5

Table 8. Proposed Relationships of Conformation of Oxytocin to Biological Activity at Uterine Receptor.

Residue	Proposed Conformational Property	Biological Consequence
Tyr-2	Side chain oriented over 20-membered disulfide ring of hormone	Essential for full efficacy
Ile-3	At corner of a β-turn or C_7 turn	Affects potency only
Gln-4	At corner of a β-turn or C_7 turn	Affects potency only
Asn-5	Side chain oriented to "interact" with side chain of Tyr-2	Essential for full efficacy
Pro-7	At corner of a reverse-turn	Affects potency only

the naturally occurring peptides at the four corner positions of the two β turns in the hormone. Side chains of residues located at corner positions are exposed and possess maximal structural freedom. Therefore, conformational consideration (Walter et al., 1971) suggest that modifications at these four positions could yield hormone analogues in which one or more of the biological activities of oxytocin were highly accentuated in terms of potency relative to other activities characteristic of these hormones. The side chain of proline in position 7 is one of the binding elements which is involved in the recognition and binding of the hormone by the uterotonic receptor. This observation initiated a series of studies in which the possibility was explored that a residue bearing unsaturated side chain with its deformable electron clouds and with its ability to undergo π-π interaction may contribute more strongly to binding (provided a steric fit at the receptor can be achieved) than the neutral, aliphatic side chain. If this argument is correct the introduction of the double bond into an aliphatic side chain which has been identified as a binding site, may increase the affinity of the resultant analogue. Consequently, the [7-L-$\Delta^{3,4}$-Pro]OT was synthesized (Moore et al., 1977). Since the formal substitution of the NH_2-terminal amino group by a hydrogen atom (Ferrier et al., 1965) or a hydroxyl group (Walti and Hope, 1972) can yield analogues with enhanced uterotonic potency, [1-β-mercaptopropionic acid, 7-L-$\Delta^{3,4}$-Pro]OT (Moore et al., 1977) and [1-L-α-hydroxy-β-mercaptopropionic acid, 7-L-$\Delta^{3,4}$-ΔPro]OT (Moore et al., 1977) also were synthesized and studied. The analogue [7-$\Delta^{3,4}$-Pro]OT had twice the uterotonic potency of OT

Table 9. Comparison of Biological Activity of Oxytocin, Deamino-Oxytocin, and Hydroxy-Oxytocin with the Corresponding Analogues Containing L-3,4-Dehydroproline in Position 7.

Peptide	Uterotonic (Rat)
[$\Delta^{3,4}$-Pro^{7}]oxytocin	1071 ± 59
Deamino[$\Delta^{3,4}$-Pro^{7}]oxytocin	1066 ± 95
Hydroxy[$\Delta^{3,4}$-Pro^{7}]oxytocin	880 ± 180
Oxytocin	546 ± 18
Deamino-oxytocin	803 ± 36
Hydroxy-oxytocin	1542 ± 18

(Table 9). The results from the log dose vs. response curve indicates that the analogue possesses a higher affinity, but the same intrinsic activity. The enhanced uterotonic potency of [$\Delta^{3,4}$-Pro^7]OT is apparently not due to any change in its overall lipophilicity as the analogue and oxytocin have almost identical R^f's on partition and thin-layer chromatography. This seems to support the idea that introducing a deformable electron cloud into a binding element could increase the affinity of the analogue for the receptor. It is also possible that the introduction of the double bond which would be expected to restrict the rapid interconversion of the pyrrolidine ring of the proline residue between various ring-puckered forms (Deslauriers et al., 1975) changes the overall conformation in such a manner as to provide a topology which is more favorable for receptor interactions. It was previously observed (Stahl and Walter, 1977) that the effect of modifications in binding elements when combined with changes at positions other than binding elements may not be additive. The activities found for the disubstituted analogues deamino[Δ^3-Pro^7]OT and hydroxy-[Δ^3-Pro^7]OT when compared to the three corresponding singly substituted analogues reinforce this view (Table 9).

Consistent with the proposal that modifications of the 4 corner positions (β-turns) would lead to the most dramatic as well as selective alterations of the biological activity profile of the hormone (Walter et al., 1968); oxytocin analogues with substitutions in position 4 (du Vigneaud et al., 1966) or 7 (Walter et al., 1976, and references therein) show a marked dissociation of the smooth muscle and

antidiuretic activities. Noteworthy is the high ratio of rat uterotonic to antidiuretic activities. From a conformation-activity viewpoint, substitutions at more than one position in oxytocin (each substitution involving only corner position of the β turns in the hormone) should result in selective modifications of the biological activity profile that reflect a summation of the changes seen with the individual monosubstituted compounds. However, substitutions at non-corner positions should not show this kind of selective addivity. In light of this assumption, the high oxytocic activity of [Thr^4]OT (Manning et al., 1970) and the negligible antidiuretic activity of [Gly^7]OT (Bodanszky and Both, 1968) are noteworthy (Table 10). The synthesis and pharmacological study of [Thr^4, Gly^7]OT (Lowbridge et al., 1977) appeared worthwhile. The result of chemically changing two corner residues of the same oxytocin analogue can be seen as a linear combination of the biological activity. Values of the relevant monosubstituted analogues, using oxytocin as a reference and calculating the percent of biological activity for a monocorner substituted analogue and then linearly combining it with that of another analogue one obtains at least in this series a first approximation of the activity of the multisubstituted compound. [Thr^4]OT has 169% of the uterotonic activity of oxytocin and [Gly^7]OT has 12%; therefore, [Thr^4, Gly^7]OT would be expected to have 20% of oxytocic activity (110 u/mg) of oxytocin. As shown in Table 11 this trend is observed. Indeed, not only are the effects additive, but they appear to be synergistic as well. The analogue [Hmp^1, Thr^4, Gly^7]OT has an oxytocic to antidiuretic ratio which is an order of

Table 10. Biological Activities of Multisubstituted Oxytocins Compared with Pertinent Monosubstituted Oxytocins.

Peptide	Oxytocic (Rat)	Antidiuretic (Rat)
Oxytocin	546 ± 18	2.7 ± 0.2
[Thr^4, Gly^7]oxytocin	270 ± 10	0.002 ± 0.0008
[Hmp^1, Thr^4, Gly^7]oxytocin	337 ± 23	0.048 ± 0.005
[Hmp^1]oxytocin	1542 ± 18	40.3 ± 2.4
[Thr^4]oxytocin	923 ± 95	1.8 ± 0.3
[Gly^7]oxytocin	65 ± 2	0.001

Table 11. Approximate Oxytocic-Antidiuretic Ratios of Oxytocin and Analogues.

Peptide	Oxytocic/ Antidiuretic Ratio
Oxytocin	200
[Thr^4]oxytocin	500
[Gly^7]oxytocin	6500
[Thr^4, Gly^7]oxytocin	135000
[Hmp^1, Thr^4, Gly^7]oxytocin	7000
[Hmp^1]oxytocin	40

Ratios are based on the potencies reported in Table 10.

magnitude larger than either monosubstituted material (Table 10). However, the introduction of a hydroxyl group in place of primary amino group of the cysteinyl moiety in position 1, a residue not in a corner position of the proposed β-turns for oxytocin results in a non-specific increase of all biological activities measured. Therefore, modification of corner positions of β-turns of the peptide backbone result in a first order approximation that the biological activities are additive. In contrast, modification of residues which are not in corner positions result in non-linear biological relationships.

The 2-position of oxytocin has long been postulated as critical to both agonist (Walter et al., 1971) and antagonist (Meraldi et al., 1975) activity. In the cooperative model, Walter proposed that placement to the tyrosine aromatic side chain over the 20-membered disulfide ring of oxytocin was critical to agonist activity. This view was supported by the observations that [D-Tyr^2]OT was a partial agonist (Hruby et al., 1979) and that in the antagonist [Pen^1]OT the conformation of the Tyr side chain groups, which would place the aromatic group over the 20-membered ring, was excluded (Hruby, 1981). Furthermore, the observation that though [Leu^2]OT has only about 1/1000 the potency of oxytocin as an agonist [Pen^1, Leu^2]OT is about 3 times more potent than [Pen^1]OT as an antagonist could be explained by the conformational finding that the Leu^2 side chain group in [Pen^1, Leu^2]OT could assume a pseudo-axial conformation over the 20-membered ring, but conformational compensation fixes the Asn^5 and half-Cys^6 side chain groups in a manner that did not permit transduction (Table 12). Interestingly, the

Table 12. Inhibitor Potencies (pA_2) for [Pen^1]Oxytocin Analogues and Agonist Potencies for Corresponding Agonists in the Rat Uterus Assay.

Peptide	pA_2	Oxytocic Activity (USP) (units/mg)
Oxytocin		546 ± 18
[Pen^1]oxytocin	6.89	
[Leu^2]oxytocin		0.5
[Pen^1, Leu^2]oxytocin	7.14	
[cLeu^2]oxytocin		4.9 ± 0.5
[Pen^1, cLeu^2]oxytocin	6.70	
[Thr^4]oxytocin		976 ± 25
[Pen^1, Thr^4]oxytocin	7.55	
[$\Delta^{3,4}$-Pro^7]oxytocin		1071 ± 59
[Pen^1, $\Delta^{3,4}$-Pro^7]oxytocin	Prolonged inhibitory activity (<7.5)	

bicyclic analogue [Pen^1, cLeu^2]OT is a much weaker antagonist than [Pen^1, Leu^2]OT or even [Pen^1]OT an observation consisent with the fact the c-Leu^2 side chain group conformation is such that it cannot be placed over the 20-membered ring. However, it is very interesting that [cLeu^2]OT is 10 times more potent as an agonist than [Leu^2]OT and is a full agonist. Conformational and dynamic studies (Hruby et al., 1982) indicate that [cLeu^2]OT and [Pen^1, cLeu^2]OT have very similar conformational and dynamic properties to oxytocin and [Pen^1]OT, respectively (Table 12). These results provide further evidence for our suggestion (Hruby, 1981) that oxytocin antagonists at the uterine receptor utilize different structural and conformational features of their own and of the receptor for interacting with the receptor. The β-branching of the residue in the 4-position of oxytocin by use of the Thr residue almost doubles potency (Manning et al., 1970); its effect on inhibitor potency is even greater, increasing this about seven times relative to [Pen^1]OT. [Pen^1, Thr^4]OT has very similar properties as [Pen^1]OT. In terms of position 4, the presence of either lipophilic or uncharged hydrophilic substituents on the β carbon atom of the amino acid residue in position 4 leads to retention of differing degrees of oxytocin-like activities and, furthermore, optimal oxytocic activities are afforded by the possession of both characteristics i.e. lipophilic and hydrophilic by the group or groups attached to the β-carbon. By comparing the rat uterus activities of [Ser^4]OT (Berde and Boissonnas, 1968) and [Asn^4]OT (Berde and Boissonnas, 1968) with those of [α-aminobutyric acid]OT (Flouret and du Vigneaud, 1969) it can be seen that substitution of

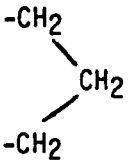
either a hydroxyl group or a carboxamide group for a methyl group on the β-carbon atom of the side-chain leads to increased oxytocic activity in both instances (Table 13), clearly indicating the predominance of hydrophilic versus lipophilic characteristics in mediating the peptide-receptor response.

Furthermore, two methyl groups attached to the β-carbon as in [Val^4]OT (Flouret and du Vigneaud, 1969) gives an analogue which is twice as active in the rat uterus assay system as one in which the β-carbon has only one methyl group as in [4-α-aminobutyric acid]OT or one ethyl group as in [NVal^4]OT (Flouret and du Vigneaud, 1969). However, the replacement of one of the methyl groups of [Val^4]OT by an ethyl group as in [Ile^4]OT (Flouret and du Vigneaud, 1969) leads to a loss of 3-fourth of oxytocic activity. Likewise, either a propyl radical as in [NLeu^4]OT (Flouret and du Vigneaud, 1969) or the isopropyl radical as in [Leu^4]OT (Flouret and du Vigneaud, 1969) attached to the β-carbon further diminishes activity (Table 13). This graded loss of activity, when one or both of the mehtyl groups on the β-carbon of [Val^4]OT is replaced by either hydrogen or larger alkyl groups would tend to indicate that steric and electronic factors may be important for the manifestation of optimal hormone-receptor interaction.
Steric hindrance appears to be responsible for the observed decrease in rat uterus activity when a group containing more than two carbon atoms is attached to the β-carbon at the residue in position 4. The higher activity of [Val^4]OT than that of either [NVal^4]OT on [4-α-aminobutyric acid]OT may be due to the greater positive inductive effect of the two

Table 13. The Effect of the Structure of the Side Chain of Amino Acids Substituted in the 4-Position of Oxytocin on Rat Uterus (RU) Activities in U/mg.

Amino Acid in 4-Position	Structure of Side Chain	RU
Threonine	$-CH(CH_3)-OH$	920
Glutamine	$-CH_2-CH_2-C(=O)-NH_2$	450
Serine	$-CH_2-OH$	195
Valine	$-CH(CH_3)-CH_3$	140
Asparagine	$-CH_2-C(=O)-NH_2$	108
α-Aminobutyric Acid	$-CH_2-CH_3$	72
Norvaline	$-CH_2-CH_2-CH_3$	61
Ornithine	$-CH_2-CH_2-CH_2-NH_2$	58
N^4-Methyl-asparagine	$-CH_2-C(=O)-NH-CH_3$	41
Isoleucine	$-CH(CH_3)-CH_2-CH_3$	37

Table 13 -- Continued

Amino Acid in 4-Position	Structure of Side Chain	RU
Alanine	$-CH_3$	36
Norleucine	$-CH_2-CH_2-CH_2-CH_3$	21
Glycine	$-H$	3
Glutamic Acid	$-CH_2-CH_2-\overset{\overset{O}{\|\|}}{C}-OH$	1.5
Proline		0.007

methyl groups at the secondary β-carbon of valine as compared to the weaker inductive effects of the methyl and ethyl groups of the primary β-carbons of α-aminobutyric acid and norvaline, respectively. When comparing the oxytocic activities of [Ser^4]OT with those of [Asn^4]OT it is clear that the hydroxyl group exerts a greater effect than the carboxamide group in mediating the peptide-receptor response. Furthermore, the hydroxyl group exerts more effect than the two methyl groups of [Val^4]OT whereas the carboxamide group of [Asn^4]OT does not. It is evident then that the hydroxyl group possesses to a greater degree than the carboxamide group these characteristics which can be loosely defined as hydrophilic (hydrogen binding ability, polar bonding, but in this case non-ionized) which are required in the side chain at position 4 to give a maximal peptide-receptor interaction. From the foregoing, therefore, one could reason that oxytocin and [Thr^4]OT are about equal as far as the lipophilic requirement at position 4 is concerned, with the methylene group on the β-carbon of the glutamine side chain and the methyl group on the β-carbon of the threonine side chain have an equal amount of lipophilicity. In a qualitative sense, therefore, the twofold increase in oxytocic activity of [Thr^4]OT over oxytocin can be seen as being due to the aformentioned predominant hydrophilicity of the hydroxyl groups as compared to the carboxamide group in the peptide-receptor interaction.

The steric and electronic requirements at position 4 would also appear to be better fulfilled by the presence on the common β-carbon of the relatively small methyl and hydroxyl groups of threonine rather than

the larger methylene carboxamide group of glutamine. In summary, it can be seen by analysis of the results to date on the rat uterus activities of 4 substituted analogues of oxytocin that:

1. Optimal activities are afforded by the presence of both hydrophilic and lipophilic grous in the side chain of the amino acid at position 4.
2. Steric hindrance by lipophilic groups containing more than two carbon atoms lends to diminished activity.
3. The hydrophilic quality of the side chain at position 4 exerts a greater effect than the optimal lipophilic quality in contributing to the rat uterus potency of the peptide.
4. Of the hydrophilic groups that have been examined to date, the hydroxyl and carboxamide are by far the most influential in endowing a peptide with rat uterus contracting ability indicating the requirement for a non-ionized hydrophilic group.
5. The hydroxyl group exerts a greater effect than the carboxamide group in bringing about increased rat-uterus contracting ability.
6. Since the threonine residue incorporates to a greater degree than glutamine residue all of the features 1-5, it is reasonable to speculate that because of this, its substitution for glutamine in 4 position of oxytocin has brought about the observed enhanced potencies and such alteration in the position 4 seems to be valuable in enhancing affinity to the uterine receptor.

Sometime ago it was shown that substitution of Pro^7 in oxytocin by 3,4-Dehydroproline provided an agonist analogue with double the potency of the native hormone, since this position is believed to be important primarily for recognition of the receptor and the 3,4-double bond renders the proline residue more conformationally rigid. Examining this structural modification on antagonist potency in [Pen^1]OT shows enhanced antagonist potency, but even more interestingly, this peptide and others had greatly prolonged activity (Table 12).

Examination of conformational, dynamic properties, and biological activity of agonist and antagonist series have led to the conclusion that oxytocin agonist and antagonist analogues use quite different structural and conformational features for receptor binding (Hruby and Mosberg, 1981). A summary of some of the data is given in Table 14 where the biological effect of substitution of particular amino acid into agonist and antagonist analogues is given. Note that while [Leu^2]OT is about 1200 fold less potent as an agonist than oxytocin (Hruby et al., 1967), [Pen^1, Leu^2]OT is about 3 fold more active as an antagonist than [Pen^1]OT (Hruby et al., 1979). Similarly, [Orn^8]OT is about 13 fold less potent than oxytocin (Boissonnas et al., 1956), but [dPen^1, Orn^8]OT is about 10 fold more potent than [dPen^1]OT and [Pen^1]OT (Sawyer et al., 1980) as an antagonist. Apparently, the agonist and antagonist series possess quite different structure requirements at position 2 and position 8 for interacting with the uterine receptor.

The major thrust of this research has been conformational restriction via side chain moieties. The side chain moieties or

Table 14. Differential Biological Effects of Specific Structural Modification on Oxytocin Agonist and Antagonist Potencies.

Peptide	Agonist Potency U/mg	Antagonist Potency pA_2
Oxytocin	546 ± 18	6.86
[Pen^1]oxytocin		
[Leu^2]oxytocin	0.44	
[Pen^1, Leu^2]oxytocin		7.14
[Orn^8]oxytocin	42 ± 5.0	
[dPen^1, Orn^8]oxytocin		7.89
[β-Mpa^1]oxytocin	802 ± 36	
[dPen^1]oxytocin		6.94

clusters of side chain moieties appear to be the major sites of interaction of the peptide hormone with complementary groups on the receptor. Thus, conformational restriction at such groups to give topological structures complementary to the receptor should permit strong interactions leading to more potent and more specific inhibitors. From earlier discussions, incorporation of $\Delta^{3,4}$-Pro and $\Delta^{4,5}$-leucine into position 7 and 8 of oxytocin and Pen^1-oxytocin should be quite interesting (Table 15), since both positions are important to binding. It might be that dehydroamino acids which are highly lipophilic, but more restricted, might facililate binding of the antagonist to the receptor. The first two analogues [L-$\Delta^{4,5}$-$Leucine^3$]OT and [L-$\Delta^{4,5}$-$Leucine^8$]OT (Table 15) were made to observe whether or not the incorporation of dehydroleucine amino acid into two binding sites of oxytocin leads to a similar type of biological activity as observed for [$\Delta^{3,4}$-Pro^7]OT. Based in the Discussion presented, I expect both of the above analogues to be agonists with 2 times the affinity of oxytocin for uterine receptor, but the same intrinsic activity. With respect to the third analogue [Leu^2, Thr^4, $\Delta^{3,4}$-Pro^7, $\Delta^{4,5}$-Leu^8]OT the synergistic and additivity characteristic of multisubstituted analogues at β-turn of oxytocin predicts this analogue to also be agonist with enhanced affinity for uterine receptor due to enhanced lipophilicity of side chains of amino acids and hence the entire molecule (Table 15). The fourth analogue [Pen^1, $Tyr(OMe)^2$, Thr^4, $\Delta^{3,4}$-Pro^7, $\Delta^{4,5}$-Leu^8]OT, has the β,β-dimethyl substitution on cysteine in position one which not only restrict the dihedral angle through the steric effect of quaternary

Table 15. Four New Unsaturated Analogues of Oxytocin Presented in this Thesis.

Peptide	Conformational Restriction	Predicted Activity
[L-$\Delta^{4,5}$Leu^3]OT	Unsaturation	Agonist/ greater affinity
[L-$\Delta^{4,5}$Leu^8]OT	Unsaturation	Agonist/ greater affinity
[Leu^2,Thr^4,Δ^3Pro^7,Δ^4Leu^8]OT	Unsaturation	Agonist
[Pen^1,Tyr(OMe)^2,Thr^4,Δ^3Pro^7,Δ^4Leu^8]OT	Gem-dimethyl and Unsaturation	Antagonist, Prolonged Activity

carbon, but also restrict the 20-membered ring through the transannular geminal dimethyl effect. Furthermore, the alkylation of tyrosine side chain leads to antagonistic activity as a result of its binding differently to the oxytocin uterine receptor than an agonist such that transduction cannot occur (Table 15). This may also affect the relative relationship of the side chain groups of Tyr^2 and Asn^5 which is important for transduction to occur. The remaining modifications in this analogue at position 4, 7, and 8 are expected to enhance the affinity of resulting analogue for the uterine receptor whereas the modificiations in position 1 and 2 should minimize biological message. Hence, such a combination should lead to an antagonist. Depending on the inhibitor and/or agonist potencies of these compounds, we will examine the conformational and dynamic properties of the most active or inactive of these compounds so as to further refine our model for the binding message of [Pen^1]OT antagonist series at the uterine receptor.

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVES

Considerable progress that has been made in the design and synthesis of conformationally restricted (semi-rigid) peptide hormone analogues with significant biological activities including superagonist and antagonist activties, prolonged or potentiated activity, and even oral activity, suggest that a new era in peptide design and application has begun. Despite all the progress in developing methods for examining the conformational and dynamic properties of peptides in solution, much of the information obtained has been of little use in predicting which peptide analogues or derivatives should be synthesized for specific biological or medical uses, or for obtaining insights into the conformational or other physical-chemical basis for biological activity. In part this is due to the complexity of the in vivo system, and the fact that very little is known about the specific chemical-physical properties of hormone receptor.

Progress in the future will greatly depend on the ingenuity of the chemist in designing specific semi-rigid conformational features that are important to biological activity (especially binding and transduction), and on the skills of the synthetic peptide chemist in incorporating these structures into peptides.

Such conformational restriction can be expected to have the following advantages:

1. Increased stability in biological systems such as enzymatic stability and oral activity.
2. Increased specificity for a particular receptor.
3. Amenability to more precise conformational evaluation.
4. Increase probability that conformational properties at the receptor, and in aqueous solution will agree.
5. A more rational basis for the design of new analogues to test conformation-activity hypotheses and to examine specific conformational effects of structural modification.

In peptide hormones with multiple binding elements such as oxytocin it should not be surprising to find that different receptors, exhibit different sensitivities to chemical modifications in the binding elements. Peptides with such interesting activity profile are potentially of great therapeutic value. In fact, oxytocin has been universally accepted and widely used in obstetrical practice; however, like all drugs, risks are associated with its use. In recent literature, the problems of water intoxication (a result of the natural natuiretic effect of oxytocin), venospasm, and increased incidence of neonatal jaundice have received particular attention.

Oxytocin is supplied as pitocin for intravenous use and as its citrate for buccal administration. The product is also marketed as syntocin for injection or as nasal spray, which is one of the first

examples of solid phase derived synthetic polypeptide that is licensed for clinical use.

The suggestion that peptide hormones may be useful as drugs is not a new one. Peptide hormones have several characteristics that make them more useful as drugs than the drugs now available.

1. Because peptide hormones contain amino acids as their primary sequence, the ease of enzymatic inactivation and excretion from the body may produce minimal or no side effects.
2. More importantly, peptide hormones are extremely site-specific, unlike other drugs, they interact with primarily one tissue receptor.
3. This tissue specificity prevents the hormone from interacting with other body tissues, reducing the probability of side effects or toxicity.

Thus the peptide hormones may be more useful in treating a physiological state than presently available drugs.

However, there are several problems associated with use of peptide hormones as drugs in terms of _in vivo_ activity, potency, and specificity.

Hormone action is a very complex process. The peptide interacts with numerous molecules or sites such as transport barriers, enzymes, or binding proteins as well as the specific receptors at which the response is initiated. The response to a given dose or concentration of peptide is a complex function of all these interactions, and since a structural change may affect any or all of them, the recorded activity is obviously

a complex result to be interpreted with due caution (Hofmann et al., 1963; Rudinger et al., 1964).

There is a discrepancy that exists between the activity of peptide hormone analogues *in vivo* and *in vitro*. For example, [Tyr(OMe)^2]OT analogue (Rudinger et al., 1972) under *in vitro* condition particularly in media deficient in Ca^{+2} and Mg^{+2}, acts as an effective antagonist of uterine response to oxytocin. In physiological media, and *in vivo*, it can act as partial or full agonist. Analogues with dimethyl substitutions on the β-carbon of the 1-hemicysteine(1-penicillamine analogue) or (1-β-mercaptopropionic acid) (1-deaminopenicillamine) analogue serve as an effective *in vitro* antagonist (Schultz et al., 1966), but they have mixed agonistic and antagonistic activities *in vivo* (Chan et al., 1967). When the size of the dialkyl substituents was enlarged from dimethyl in [dPen^1]OT to diethyl (Vavrek et al., 1972) and to cyclopentamethylene (Nestor et al., 1975) groups, the *in vitro* antagonistic activity increases. These analogues are not agonists *in vivo*, and act as effective antagonists of the uterine response to oxytocin (Lowbridge et al., 1979). Substitution of 4-Thr in antagonist series clearly enhances the antagonistic potencies of 1-deamino-oxytocin, d(Et)$_2$OT or [1-β-mercapto-β,β-diethylpropionic acid]OT, and d(CH$_2$)$_5$OT or [1-β-mercapto-β,β-pentamethylene]OT. Antagonistic potencies of each analogue is approximately doubled *in vitro* and *in vivo* assays by 4-Thr substitution. Although neither [dPen^1]OT or [Tyr(OMe)^2]OT are pure *in vivo* antagonists the combination of changes in [dPen^1, Tyr(OMe)^2]OT

results in disappearance of *in vivo* agonistic activity and the production of an effective antagonist of uterine response.

In addition to showing pure *in vivo* antagonist or agonist activity oxytocin analogues must be reasonably potent. Otherwise much higher doses will be needed to completely block the response to endogenous oxytocin. Otherwise, this will limit their usefulness as pharmacological tools and their possible promise as therapeutic agents for managing premature or overly vigorous labor.

The events following the peptide-receptor interaction in which the peptide no longer participates can also affect the activity of the hormone analogue qualitatively and quantitatively, and this is one area of hormone-receptor interaction which is not well understood. But, in general: 1. Enzymatic stability, and hence prolonged *in vivo* biological activity; and 2. Specificity for one receptor over another receptor are two effects of particular interest for the design of peptides with potential medicinal applications.

The physicochemical basis for peptide hormone action in terms of the conformation and dynamics of these compounds in solution and at the biological receptor, are at an early stage of development. Despite the fact that half of the system, i.e. the receptor is not available in purified form, recent developments of high-field fourier-transform NMR instruments, rapid and flexible synthetic methods, and more careful attention to the design and utilization of bioassay data have made it possible to accomplish much.

Conformational and dynamic properties which are important to binding process and which prevent transduction have been identified using conformationally restricted analogues. Based on the results obtained it is possible to propose models for antagonist activity at the rat uterine receptor. These models further suggest new analogues which may be more potent antagonists, but also illustrate the many gaps which exist in our understanding of the complementarity between the hormone and the receptor in the bound state. The ultimate goal is to be able to design peptide hormone analogues in terms of their conformational and dynamic properties at the receptor. This will require a thorough understanding of the specific stereoelectronic and conformational properties of peptide hormone at a particular receptor, and how and why these vary at different receptors. Though this is a challenging task it is the only approach that can provide a rational approach to the design of drugs related to peptide hormone function.

This investigation was an attempt at giving a brief survey of the practical results attained so far, the focus in this thesis, and expected future trends in peptide chemistry. In this regard some future analogues of oxytocin peptide which could provide more information on the mode of hormone-receptor interaction and possibly exhibit enhanced <u>in vivo</u> antagonistic properties are as follows:

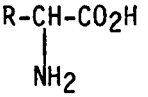
1. [Pen^1, $\Delta^{2,3}$-Phe^2, Thr^4, $\Delta^{3,4}$-Pro^7, $\Delta^{4,5}$-Leu^8]OT
2. [Pen^1, Tic^2, Thr^4, $\Delta^{3,4}$-Pro^7, $\Delta^{4,5}$-Leu^8]OT
3. [dPen^1, Tic^2, Thr^4, $\Delta^{3,4}$-Pro^7, $\Delta^{4,5}$-Leu^8]OT

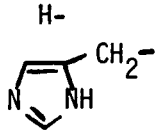
These analogues should exhibit more affinity toward the uterine receptor, and also would be expected to be conformationally more restricted.

APPENDIX A

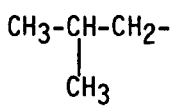
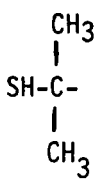
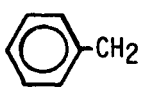
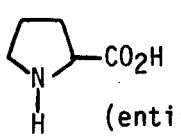
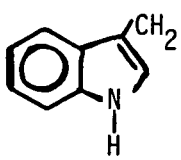
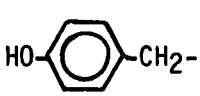
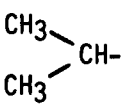
LIST OF ABBREVIATIONS

Amino Acids

General Structural Formula: 

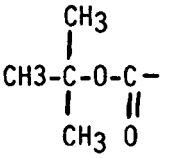
Name	R-	Abbreviation
Alanine	CH_3-	Ala
Arginine	$H_2N-C(=NH)-NHCH_2-CH_2-CH_2$-	Arg
Asparagine	$H_2N-C(=O)-CH_2$-	Asn
Aspartic Acid	HO_2C-CH_2-	Asp
Cysteine	$HS-CH_2$-	half Cys, Cys(SH)
Cystine	$-CH_2-S-S-CH_2-$	Cys, Cys⌜Cys⌝
α-Aminobutyric Acid	$H_2N-CH_2-CH_2$-	α-Abu
Glutamic Acid	$HO_2C-CH_2-CH_2$-	Glu
Glutamine	$H_2N-C(=O)-CH_2-CH_2$-	Gln
Glycine	H-	Gly
Histidine		His
Isoleucine	$CH_3-CH_2-CH(CH_3)$-	Ile

APPENDIX A--Continued

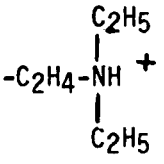
Name	R-	Abbreviation
Leucine		Leu
Lysine	$H_2N-CH_2-CH_2-CH_2-CH_2-$	Lys
Norleucine	$CH_3CH_2CH_2CH_2-$	Nleu
Norvaline	$CH_3CH_2-CH_2-$	NVal
Methionine	$CH_3-S-CH_2CH_2-$	Met
Ornithine	$H_2N-CH_2CH_2-CH_2-$	Orn
Penicillamine		Pen
Phenylalanine		Phe
Proline	 (entire structure)	Pro
Tryptophan		Trp
Tyrosine		Tyr
Valine		Val

APPENDIX A -- Continued

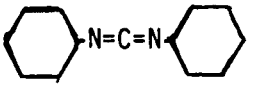
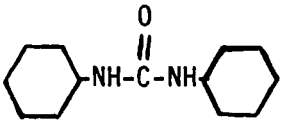
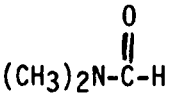
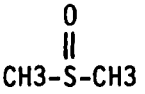
Amino Acid Protecting Groups

Protecting Group	Structure	Abbreviation
p-Methylbenzyl		Bzl
Tert-butyloxycarbonyl		t-Boc

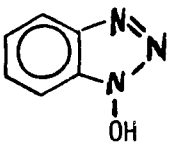
Ion Exchange Resins

Diethylaminoethyl		DEAE

Reagents and Solvents

Acetic Acid	CH_3COOH	HOAc
n-Butanol	$CH_3CH_2CH_2CH_2OH$	n-BuOH
Dicyclohexylcarbodiimide		DCC
Dicyclohexylurea		OCU
Dimethylformamide		DMF
Dimethylsulfoxide		DMSO

APPENDIX A -- Continued

Protecting Group	Structure	Abbreviation
Ethyl Acetate	$CH_3-\overset{\overset{O}{\|\|}}{C}-OCH_2CH_3$	EtOAC
Hydroxylbenzotriazole		HOBT
Hydrochloric Acid	HCl	
Methylene Chloride	CH_2Cl_2	
Pyridine		Pyr
Sodium Chloride	NaCl	
Sodium Hydroxide	NaOH	
Triethylamine	$(CH_3CH_2)_3N$	Et_3N
Trifluoroacetic Acid	CF_3COOH	TFA
Acetonitrile	CH_3CN	

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