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THE SYNTHESIS OF POLYPEPTIDES FOR X-RAY STRUCTURE DETERMINATION, PHARMACOLOGICAL AND RELATED STUDIES

by

Clark William Smith

A Dissertation Submitted to the Faculty of the DEPARTMENT OF CHEMISTRY

In Partial Fulfillment of the Requirements For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

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1973
I hereby recommend that this dissertation prepared under my direction by Clark William Smith entitled THE SYNTHESIS OF POLYPEPTIDES FOR X-RAY STRUCTURE DETERMINATION, PHARMACOLOGICAL AND RELATED STUDIES be accepted as fulfilling the dissertation requirement of the degree of DOCTOR OF PHILOSOPHY

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TO SYLVIA AND MICHAEL
This work was supported in part by Grant No. AM-14062 from the U. S. Public Health Service. All optically active amino acids are of the L variety. The symbols for the amino acid residues follow the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature. The author thanks Dr. Victor J. Hruby for his patient and enthusiastic support of this project. The author thanks Dr. Martha F. Ferger for the oxytocic, avian vasodepressor, and rat pressor assays done on the 4-tocin and [Ser^4]-tocin compounds. The author thanks Dr. Mac E. Hadley for all of the MRIF and milk ejecting assays. The author also thanks Dr. Joseph La Point for the oxytocic assays performed on mesotocin. Finally, thanks are in order to Dr. Charles Groginsky and Mr. Peter Gitu for the amino acid analyses.
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ABSTRACT

The objective of this investigation was the synthesis of polypeptides related to the neurohypophysial hormones in order to study further the structure-activity relationships of these hormones. It was thought that the synthesis of crystalline polypeptides, whose three dimensional structure could be determined by X-ray diffraction techniques, would give additional insight into the structure-activity relationships.

Three derivatives related to the carboxyl terminal end of oxytocin were synthesized. These were \( \text{D-BrBzl-Pro-Leu-Gly-NH}_2 \cdot \text{HCl} \), \( \text{Bz(Br)-Pro-Leu-Gly-NH}_2 \), and \( \text{Cys(D-BrBzl)-Pro-Leu-Gly-NH}_2 \). Crystals suitable for X-ray diffraction were obtained for the first two derivatives. Compound \( \text{D-BrBzl-Pro-Leu-Gly-NH}_2 \cdot \text{HCl} \) presented some special crystallographic problems and its structure has not been solved as yet. Compound \( \text{Bz(Br)-Pro-Leu-Gly-NH}_2 \) looked more promising and work is underway on its structure at present.

Deaminotocinoic acid and deaminotocinamide, the ring moieties of deamino-oxytocin, were also prepared. Although the crystallinity of these peptides was poor, they possessed interesting pharmacological activities. Deaminotocinoic acid and deaminotocinamide exhibited 3.7 ± 0.3 units/mg and 34.2 ± 3.0 units/mg of oxytocic activity, respectively. Neither showed avian vasodepressor activity.

The pharmacological activity exhibited by these compounds prompted the synthesis of the ring moieties contained in the
neurohypophysial hormones isotocin and glumitocin. Using the stepwise active ester method, [Ser⁴]-tocinoic acid, [Ser⁴]-tocinamide, [Ser⁴]-deaminotocinoic acid, and [Ser⁴]-deaminotocinamide were synthesized by classical solution techniques. The four compounds were found to be void of oxytocic, avian vasodepressor, and rat pressor activities. An inhibition of oxytocin by [Ser⁴]-tocinamide and [Ser⁴]-deaminotocinamide in the oxytocic assay was shown to be about 1/30 as great as that of deamino-penicillamineoxytocin. In preliminary experiments [Ser⁴]-tocinoic acid and [Ser⁴]-tocinamide failed to inhibit the release of melanophore stimulating hormone (MSH). These two compounds showed a weak milk ejecting activity about 1/10,000 as great as deamino-oxytocin.

The MSH release inhibition reported for the tocin rings prompted a synthesis of mesotocin, the naturally occurring neurohypophysial hormone of amphibians. Since these animals are so obviously affected by MSH, a study of how mesotocin affects MSH release in amphibians was desirable.

During the course of these syntheses several innovations in current techniques were studied. These innovations included the preparation of a new S protecting group for cysteine (the 3,4-dimethylbenzyl group), the use of macroreticular resin for solid phase work, and the partially successful attachment of cysteine to a solid phase resin by a thioether bond.
CHAPTER 1

INTRODUCTION

The objective of this investigation is the synthesis of polypeptides related to the neurohypophysial hormones, the study of which should lead to a better understanding of the relationship between the structural features of these polypeptide hormones and their activity. In the course of these studies several compounds were prepared which possessed interesting and unexpected pharmacological activities.

Two interdependent factors are usually considered when the structure-activity relationships of polypeptide hormones are investigated. These are the primary structure (the sequence of amino acid residues in the polypeptide backbone) and the ability of the polypeptide hormone to interact with its presumed receptor site and elicit a biological or pharmacological response. Determining the importance of the latter factor requires detailed knowledge of the conformation of the hormone and its receptor site.

Structure-activity Studies on Neurohypophysial Hormone Analogs

At the present time nine chemically related polypeptide hormones have been found in the neurohypophyses of mammals, birds, amphibians, and fish. These are oxytocin (Livermore and du Vigneaud 1949), vasopressin (Turner, Pierce, and du Vigneaud 1951), lysine-vasopressin (du Vigneaud, Lawler, and Popenoe 1953), vasotecin (Acher et al. 1960),
mesotocin (Acher et al. 1964), isotocin (Acher et al. 1962), glumitocin (Acher et al. 1965), valitocin (Acher, Chauvet, and Chauvet 1972), and aspartocin (Acher et al. 1972). All of these compounds have a 20-membered disulfide ring and a side chain of three amino acid residues terminating in a carboxamide group (see Figure 1).

In trying to establish relationships between the chemical structure and the biological activities of these hormones, over 200 analogs have been synthesized in various laboratories (Berde and Boissonnas 1968). The effect of varying amino acid residues in the ring and in the side chain, the effect of introducing D-amino acids, the effect of removing or substituting the N-terminal amino group, the effect of methylating a peptide amide group, the effect of replacing sulfur with selenium or a methylene group, and the effect of expanding or contracting the ring size have all been investigated to varying degrees. Spectroscopic studies such as nuclear magnetic resonance (nmr), circular dichroism (CD), optical rotatory dispersion (ORD), and other physical methods have been useful in gaining some insight into the possible conformations of oxytocin and the vasopressins as well as other polypeptides (Hruby in press).

**The Possible Use of X-ray Crystallography in Structure-activity Studies**

Despite this massive amount of data, it has not been possible to develop a completely satisfactory correlation between the gross structural features of these hormones and their activities. More insight into the structure-activity problem could be gained if the
Cys-Tyr-X-Y-Asn-Cys-Pro-Z-Gly-NH₂

Oxytocin:  X = Ile,  Y = Gln,  Z = Leu
Vasopressin:  X = Phe,  Y = Gln,  Z = Arg
Lysine vasopressin:  X = Phe,  Y = Gln,  Z = Lys
Vasotocin:  X = Ile,  Y = Gln,  Z = Arg
Mesotocin:  X = Ile,  Y = Gln,  Z = Ile
Isotocin:  X = Ile,  Y = Ser,  Z = Ile
Glumitocin:  X = Ile,  Y = Ser,  Z = Gln
Valitocin:  X = Ile,  Y = Gln,  Z = Val
Aspartocin:  X = Ile,  Y = Asn,  Z = Leu

Figure 1. The structure of the neurohypophysial hormones.
conformation of these hormones could be determined by X-ray crystallography. Precise information about their conformation could be obtained and a good deal more about how specific structural features affect the conformation could be learned. Correlations between structural features and similar or different conformational features found in crystalline compounds could be attempted for hormones which cannot be crystallized. Finally, attempts to discern which conformational features directly affect biological activity could be made and eventually with the knowledge of conformation of the hormones perhaps something could be said about the conformation of the receptor.

At the present time none of the naturally occurring neurohypophysial hormones have been obtained in crystalline form. Factors which prevent satisfactory crystal formation of medium-sized peptides (4-20 amino acids) are not well understood. Smaller peptides often behave like typical organic molecules and are frequently crystalline. Higher polypeptides crystallize with considerable interstitial solvent in the manner of proteins. One of the specific objectives of this investigation was to bridge the gap between these extremes and to obtain crystalline polypeptides in the size range of the neurohypophysial hormones by determining what structural features augment crystallization.

Some examples of crystalline compounds with structures similar to the neurohypophysial hormones are available. Deamino-oxytocin (Ferrier, Jarvis, and du Vigneaud 1965), (L-γ-mercaptobutyric acid)-oxytocin (Jarvis, Ferrier, and du Vigneaud 1965), and deaminopressinamide (Ferger et al. 1972) have been obtained in the crystalline form,
but no report on their X-ray structure determination has yet appeared. It should be noted that in all of these compounds the N-terminal amino group has been replaced by hydrogen.

Using these examples of crystalline peptides as a guide, several polypeptides which had a high probability of being crystalline were synthesized. These included derivatives of the side chain of oxytocin (Pro-Leu-Gly-NH$_2$), the deamino analogs of the tocin ring (deamino-tocinoic acid, $\beta$-MPA-Tyr-Ile-Gln-Asn-Cys, and deaminotocinamide, $\beta$-MPA-Tyr-Ile-Gln-Asn-Cys-NH$_2$). Interesting results obtained from pharmacological studies of the tocin ring led to the preparation of the corresponding ring moieties of isotocin and glumitocin ([Ser]$^4$-tocinoic acid, [Ser]$^4$-tocinamide, [Ser]$^4$-deaminotocinoic acid, and [Ser]$^4$-deaminotocinamide).
CHAPTER 2

CRYSTALLINE DERIVATIVES OF THE SIDE CHAIN OF OXYTOCIN

Oxytocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂, is probably the most widely studied of all the neurohypophysial hormones. The structure of oxytocin consists of a 20-membered disulfide-containing ring (see Chapter 3) and a side chain of three amino acid residues terminating in a carboxamide, Pro-Leu-Gly-NH₂ (Boissonnas et al. 1955). This tripeptide was obtained in a crystalline form which appeared suitable for X-ray structure determination. At this time the structure has not been solved due to the unusual space group and dimensions of the crystalline form though recent work (L. Reed, Department of Chemistry, The University of Arizona, private communication, 1973) suggests an X-ray structure will be forthcoming. It has been recently reported that Pro-Leu-Gly-NH₂ is effective in the inhibition of the release of melanophore stimulating hormone (MSH) from the pituitary (Celis, Taleisnik, and Walter 1971; Kastin, Schally, and Viosca 1971). It would, therefore, be very desirable to determine the three-dimensional structure of this tripeptide not only for itself, but also to be able to use the X-ray structure as a starting point to compare by spectroscopic means the conformation of the tripeptide as it exists by itself and as it exists when attached to the rest of the hormone.

The search for a derivative which would allow the tripeptide to crystallize in another form and which would at the same time aid in
solving the structure was begun. It was reported that the p-bromobenzyl-oxycarbonyl group, Z(Br) (for a list of abbreviations and the chemical formulas used, see Appendix A), gave derivatives which crystallized well (Channing, Turner, and Young 1951). Since this derivative also contains bromine as a heavy atom to use in the X-ray structure determination, it would seem to meet both criteria of a desirable derivative for Pro-Leu-Gly-NH₂.

**Synthesis of pBrBzl-Pro-Leu-Gly-NH₂·HCl**

The original goal of this synthesis was the preparation of Z(Br)-Pro-Leu-Gly-NH₂. However, elemental analysis of the final product showed the presence of chlorine and suggested the structure to be pBrBzl-Pro-Leu-Gly-NH₂·HCl. This was confirmed by mass spectral data. The difficulty arose because of the decomposition of p-bromobenzylxy-carbonyl chloride to p-bromobenzyl chloride when a vacuum distillation of the former was attempted.

**Experimental Section for the Classical Approach**

The tripeptide derivative, pBrBzl-Pro-Leu-Gly-NH₂·HCl, was synthesized in two ways. In this section is described the approach using the mixed anhydride method of peptide synthesis similar to that used in a preparation of Z-Pro-Leu-Gly-NH₂ (Cash 1961). All melting points were taken in capillary tubes and are uncorrected.

**Synthesis of p-Bromobenzyl Alcohol.** A slurry of LiAlH₄ (5.7 g, 0.15 mol) was prepared in 500 ml of anhydrous Et₂O (dried with Na). To the flask was attached a continuous extraction apparatus containing
p-bromobenzoic acid (20.0 g, 0.10 mol). The slurry was refluxed until all of the p-bromobenzoic acid had been extracted into the Et₂O (Nystrom and Brown 1947). The general procedure for working up LiAlH₄ reactions which gives filterable Al₂O₃ rather than Al(OH)₃ was used to work up the reaction mixture (Micovic and Mihailovic 1953). In this case, 5.7 ml of H₂O, 5.7 ml of 15% NaOH solution, and 17.1 ml of H₂O were added to the reaction mixture. The resulting white, granular precipitate was filtered off and washed twice with warm Et₂O. The combined filtrates were dried with CaSO₄ and the Et₂O was removed by rotary evaporation: wt 16.6 g (89%); mp 74-75.5° (lit. 76°).

**Synthesis of p-Bromobenzyl Chloride.** A solution of p-bromobenzyl alcohol (16.6 g, 0.089 mol) was prepared in 125 ml of anhydrous toluene containing N,N-dimethylaniline (12 g, 0.1 mol). This solution was added to a solution of phosgene at 0-5° over a 60-min period. The resulting solution was stirred without additional cooling overnight. A white precipitate had formed by morning. The reaction mixture was stirred under an aspirator vacuum until bubbles of phosgene ceased to evolve. The mixture was extracted with 40 ml of ice water, 30 ml of 5% HCl solution, and two more 40-ml portions of ice water. The toluene layer was separated, dried over CaSO₄, and later removed by rotary evaporation (Baizer, Clark, and Smith 1957). The product, p-bromobenzyl-oxycarbonyl chloride, spontaneously lost CO₂ when distilled at 73-75° under 0.6-0.7 torr pressure to give p-bromobenzyl chloride: wt 14.4 g (78%); mp 35-37° (lit. 39°).
Synthesis of p-Bromobenzylproline Hydrochloride. A solution of proline (0.92 g, 8 mmol) was prepared in 4 ml of 2 N NaOH. To this was simultaneously added p-bromobenzyl chloride (1.0 g, 7.1 mmol) in 4 ml of dioxane and 2.4 ml of 4 N NaOH. The resulting two-phase system was stirred for 24 hr after which only one phase remained. The dioxane was removed by rotary evaporation and the aqueous phase was extracted twice with Et₂O. To the aqueous solution solid NaCl was added until an organic component just began to separate. The solution was then cooled to -10° and was slowly made acidic with concentrated HCl. Saturated aqueous NaCl solution was added to keep the mixture from becoming completely solid. The product, which separated as a granular precipitate, was collected and washed with saturated NaCl solution until the pH of the filtrate was neutral. After drying, the product was dissolved in CHCl₃-MeOH (10:1). The solution was filtered while hot to remove NaCl. The product was isolated from the cool solution in two crops: 1st crop, 1.21 g, mp 215-216.5°; 2nd crop, 0.41 g, mp 211.5-213° (total yield, 71%); [α]²⁴°⁺⁺⁻⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊₊groupId: 9,
isobutylchloroformate (0.45 g, 3.3 mmol) in 5 ml of THF was added. After stirring for 1 hr some undissolved pBrBzl-Pro could still be distinguished. (Another precipitate also separated during this period.) An additional 0.2 g (1.5 mmol) of isobutylchloroformate was added and stirring at -10° was continued for 30 min. The starting material could no longer be seen. A solution of leucine (0.72 g, 5.5 mmol) in 5 ml of H₂O with Et₃N (1.5 ml, 10 mmol) was added and the reaction was allowed to continue without additional cooling for 48 hr. The reaction mixture was filtered to remove some undissolved leucine and the THF was removed by rotary evaporation. The reaction mixture was slowly made acidic with concentrated HCl. The product which separated was collected, washed with cold H₂O, and dried in vacuo: wt 1.8 g; mp 180-183°. This crude product was purified by recrystallization from EtOH-H₂O. It was collected, washed with EtOH-H₂O (1:1), and dried in vacuo: wt 0.86 g (64%); mp 185-187°.

Synthesis of Glycinamide Acetate. This compound (Chambers and Carpenter 1955) was prepared from glycine ethyl ester hydrochloride (14.0 g, 0.10 mol) dissolved in 50 ml of MeOH to which Et₃N (14 ml, 0.1 mol) was added. After 10 min, 200 ml of Et₂O was added and the resulting slurry cooled at 0° for 60 min. The mixture was filtered and the Et₃N·HCl (12.3 g, 89%) collected was washed with Et₂O. The combined filtrates were evaporated to a residue of straw-colored oil which was added to 150 ml of anhydrous MeOH saturated with anhydrous NH₃ at 0°. After three days the MeOH and NH₃ were removed by evaporation and the residue dissolved in 50 ml of fresh MeOH. The solution was
filtered to remove 0.6 g of diketopiperazine (compared by IR to an authentic sample) and the MeOH was evaporated off. This process was repeated without the filtration step two more times. The oily product was taken up in 30 ml of MeOH, 6 ml of HOAc was added, and EtOAc was added until the product began to separate. After cooling and slow addition of EtOAc the product was collected, washed with EtOAc, and dried in vacuo: wt 9.6 g (72%), mp 109-112\(^\circ\) (lit. 122-124\(^\circ\)). This product was very hygroscopic, requiring that it be handled under N\(_2\). The nmr showed a significant amount of H\(_2\)O as a contaminant causing the observed low mp.

**Synthesis of pBrBzl-Pro-Leu-Gly-NH\(_2\)·HCl.** A solution of pBrBzl-Pro-Leu·HCl (0.60 g, 1.4 mmol) was prepared in 5 ml of THF containing Et\(_3\)N (0.3 ml, 2.1 mmol) (the amount of base should have been 2.8 mmol at least). This solution was cooled to -14\(^\circ\) and isobutylchloroformate (0.22 g, 1.6 mmol) dissolved in 3 ml of THF was added. After 30 min, glycaminide (0.11 g, 1.4 mmol) in 1.5 ml of H\(_2\)O containing 0.3 ml of Et\(_3\)N was added and stirring was continued for 48 hr without additional cooling. A 5-ml portion of H\(_2\)O was added and the reaction mixture was slowly made acidic with concentrated HCl. The THF was removed by rotary evaporation and the solution was saturated with NaCl. An oil which had a density greater than the solution separated. The solution was decanted off and the residue triturated to a solid with Et\(_2\)O, CHCl\(_3\), and EtOAc. The residue was taken up in MeOH which was then evaporated to dryness leaving 0.72 g of solid. This was dissolved in CHCl\(_3\)·MeOH and filtered while hot to remove NaCl which had been carried
along. The product, which precipitated when the solution was cooled, was collected, washed with CHCl₃, and dried in vacuo: wt 0.18 g (25%); mp 252-255°; [α]₂₄°₅₄⁶⁻₅₄.₂° (c 0.4, H₂O). The product gave a single spot on tlc using Silica Gel-G in the system CHCl₃-MeOH (9:2). The compound showed a parent peak in the mass spectrometer at mass number 452 and showed a strong peak at mass number 36. Elemental analysis calculated for C₂₀H₃₀N₄O₃ClBr: C, 49.03; H, 6.18; N, 11.44; Cl, 7.24; Br, 16.31. Found: C, 48.80; H, 6.05; N, 11.46; Cl, 7.02; Br, 16.04.

Experimental Section for the Solid Phase Approach

The low overall yield of pBrBzl-Pro-Leu-Gly-NH₂·HCl obtained by the mixed anhydride method (11%) prompted another synthesis of this material by a second method. The solid phase method (Gutte and Merrifield 1971) was chosen as a likely alternative.

Preparation of Boc-Gly-OResin. The first amino acid, Boc-Gly, was attached to polystyrene resin 2% crosslinked with divinylbenzene and chloromethylated to the extent of 2 mmol/g (Stewart and Young 1969, p. 32). A solution of Boc-Gly (1.23 g, 7 mmol) was prepared in 15 ml of absolute EtOH, and Et₃N (0.91 ml, 6.5 mmol) and 5.00 g of polystyrene resin was added. The mixture was refluxed for 48 hr. The resin was transferred to a separatory funnel with 100 ml of CH₂Cl₂. It was allowed to stand until a distinct phase of floating resin appeared. The solvent was run off until only the floating resin phase remained. In this manner the smallest particles of resin suspended in the solvent were discarded. The resin was shaken with a fresh portion of CH₂Cl₂.
and the process was repeated a total of four times. The resin was transferred back to the Buchner funnel and the solvent was removed. After drying in vacuo the resin weighed 5.36 g. A 25-mg portion of the resin was treated with trifluoroacetic acid (TFA) to remove the Boc protecting group; then the resin was neutralized with diisopropylethylamine (DIEA). The substitution (amount of glycine attached to the resin) was determined by the aldimine test (Esko, Karlsson, and Porath 1968) as modified by Ehler (1972, pp. 39-40) and was found to be 0.53 mmol/g.

Steps of Solid Phase Synthesis. A 2.00-g portion of Boc-Gly-OResin was used for the synthesis. The sequence of treatments used on the resin is shown in Table 1. Completion of all the steps (1-14) accomplished the attachment of one amino acid residue. Steps 9-12 were performed in an additive fashion. In other words, the reagent introduced in step 10 was added to that present from step 9 and so on through step 12. In all of the other steps the reagent introduced in the previous step was removed from the resin before the next step was begun.

Coupling Steps. For the coupling of leucine to glycine a 2.5-fold excess (over the amount of glycine on the resin) of Boc-Leu (0.65 g, 2.8 mmol) was used in step 9 along with an equivalent amount of dicyclohexylcarbodiimide (DCC) (0.57 g, 2.8 mmol) in step 11 for a reaction time of 5 hr. A ninhydrin test was run after step 14 which showed the reaction to be greater than 99.4% complete (Kaiser et al. 1970).
Table 1. Sequence used in solid phase synthesis.

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Purpose</th>
<th>Solvent or reagent</th>
<th>Vol, (ml)</th>
<th>Time (min)</th>
<th>Repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>wash</td>
<td>( \text{CH}_2\text{Cl}_2 )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>deprotect</td>
<td>TFA-( \text{CH}_2\text{Cl}_2 ) - anisole (49:49:2)</td>
<td>25</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>wash</td>
<td>( \text{CH}_2\text{Cl}_2 )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>wash</td>
<td>( \text{EtOH} )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>wash</td>
<td>( \text{CHCl}_3 )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>neutralize</td>
<td>( \text{DIEA-CHCl}_3 ) (1:9)</td>
<td>25</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>wash</td>
<td>( \text{CHCl}_3 )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>wash</td>
<td>( \text{CH}_2\text{Cl}_2 )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>couple</td>
<td>Boc-amino acid- ( \text{CH}_2\text{Cl}_2 )</td>
<td>10</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>flush</td>
<td>( \text{CH}_2\text{Cl}_2 )</td>
<td>2.5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>couple</td>
<td>DCC in ( \text{CH}_2\text{Cl}_2 )</td>
<td>10</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>flush</td>
<td>( \text{CH}_2\text{Cl}_2 )</td>
<td>2.5</td>
<td>varies</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>wash</td>
<td>( \text{CH}_2\text{Cl}_2 )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>wash</td>
<td>( \text{EtOH} )</td>
<td>25</td>
<td>2</td>
<td>3</td>
</tr>
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</table>
For the coupling of pBrBzl-Pro·HCl the solvent in steps 8-13 was changed to dimethylformamide (DMF). The solution of pBrBzl-Pro·HCl (0.87 g, 2.7 mmol) was prepared in 20 ml of DMF by warming to 40°. The DCC (0.49 g, 2.4 mmol) solution was prepared in 3 ml of DMF. The reaction was run for 3 hr at 40°. A determination of the completeness of the reaction was made after step 14 using the aldimine test. The test showed that the coupling had only gone 63% toward completion.

Steps 8-14 were repeated using 0.26 g (0.8 mmol) of pBrBzl-Pro·HCl and 0.22 g (1.6 mmol) of 1-hydroxybenzotriazole which is reported to stop some side reactions during DCC couplings (Konig and Geiger 1970) in step 9 and 0.20 g (0.8 mmol) of DCC for 5 hr at room temperature in step 11. The percent completion rose to 73%. Steps 8-14 were again repeated using the same conditions as described above except the reaction was allowed to go for 17 hr. The percent completion rose to 92% and could not be improved by another repetition of steps 8-14 for 47 hr.

Removal of the Peptide from the Resin and Purification. The resin was transferred from the reaction vessel to a tared Buchner funnel, washed with CH₂Cl₂, and dried in vacuo overnight: wt 2.30 g. A weight gain of 0.51 g was possible. The resin was slurried in 200 ml of anhydrous MeOH which was then saturated with anhydrous NH₃ at 0° (Stewart and Young 1969, p. 44). The resin was stirred for 48 hr at 0°. The MeOH and NH₃ were removed by rotary evaporation and the resin was extracted with H₂O. Rotary evaporation of the aqueous solution gave 0.23 g of white solid. This was dissolved in MeOH-CHCl₃ (1:4) and filtered
to remove a few crystals of $\text{NH}_4\text{Cl}$. Adding $\text{Et}_2\text{O}$ to the solution brought down a white precipitate which was collected, washed with $\text{Et}_2\text{O}$, and dried in vacuo: wt 0.11 g (22%); mp 246-247°. The nmr was identical to the product obtained by the mixed anhydride method.

**Conclusion**

As previously mentioned the goal of the synthesis just described was $Z(\text{Br})$-Pro-Leu-Gly-$\text{NH}_2$. The low yields and other difficulties encountered were undoubtedly due to the fact that non-equivalent conditions existed in most reactions. This was especially true when bases were required because of the erroneous assumption that the derivative of proline was $Z(\text{Br})$-Pro when in fact it was $p\text{BrBzl}$-Pro·$\text{HCl}$. It was fortuitous that $p\text{BrBzl}$-Pro-Leu-Gly-$\text{NH}_2$·$\text{HCl}$ showed promise of being a good crystalline derivative. The derivative could be obtained in a form suitable for X-ray structure determination by diffusing acetone into an aqueous solution of the product (mp 261-262°). It crystallized in the space group $P2_1$ with two molecules per unit cell. Unfortunately the two heavy atoms, chlorine and bromine, occur at the same $y$ coordinate in the cell to further complicate the structure determination. At this time work has stopped on this derivative in favor of work on the derivative synthesized in the following section.

Of the two methods used to synthesize $p\text{BrBzl}$-Pro-Leu-Gly-$\text{NH}_2$·$\text{HCl}$, the solid phase method gave a better yield than the mixed anhydride method (11 vs. 22%). This yield could probably have been improved further had it been known at the time that the derivative was an $\text{HCl}$ salt and had the crude product from the resin been treated with $\text{HCl}$
to insure that the product was in that form. In addition, the product isolated from the solid phase method was free from the troublesome contamination by NaCl present in the product of the mixed anhydride method. Neither method was particularly adaptable to this synthesis because of the low solubility of the HCl salts in most organic solvents.

**Synthesis of Bz(Br)-Pro-Leu-Gly-NH₂**

When it became apparent that there were difficulties connected with solving the crystal structure of \( \text{pBrBz1-Pro-Leu-Gly-NH₂·HCl} \), consideration had to be given to an alternative derivative. Originally the Z(Br) group was chosen because it contained a heavy atom and led to crystalline derivatives (Channing et al. 1951). The benzyloxycarbonyl group is used in peptide synthesis because it affords good protection against racemization and because it can be easily removed under mild conditions. For the purpose described here only the advantages of the heavy atom and crystallinity were required. The group attached to the proline residue does not need to be removable and since proline is not easily racemized, it need not be a particularly good protecting group in this regard. These facts plus the difficulty encountered in obtaining \( \text{p-bromobenzyloxy carbonyl chloride} \) led to the choice of the \( \text{p-bromo} \) benzoyl group.

**Failure of the Mixed Anhydride Method**

The combination of conditions for the mixed anhydride method, which had been reported to give a minimum of racemization and a maximum yield (Anderson, Zimmerman, and Callahan 1967), was chosen to try to
extend Bz(Br)-Pro to Bz(Br)-Pro-Leu (see below for the synthesis of Bz(Br)-Pro.) A solution of Bz(Br)-Pro (2.82 g, 9.47 mmol) was prepared in 15 ml of EtOAc containing N-methylmorpholine (12 ml, 10.7 mmol) and cooled to -10°. To this solution was added isobutyl chloroformate (1.3 ml, 10 mmol) and the white emulsion formed was stirred 2 min. A slurry of leucine (1.4 g, 10.7 mmol) and N-methylmorpholine (1.2 ml, 10.7 mmol) in 10 ml of H₂O was added and stirring was continued for 2 hr without additional cooling. From this reaction 49% of the starting Bz(Br)-Pro (as the dicyclohexylamine salt) and 36% of the leucine were recovered. No dipeptide could be isolated.

Experimental Section

The failure of the mixed anhydride method prompted the use of the solid phase method to synthesize this derivative.

Synthesis of p-Bromobenzoylproline. A solution of proline (4.7 g, 40.8 mmol) was prepared in 21 ml of 2 N NaOH and cooled to 0°; p-bromobenzoyl chloride (10 g, 45 mmol) dissolved in 7 ml of Et₂O was added in 1 ml aliquots along with 21 ml of 2 N NaOH in 2-ml aliquots over a 30-min period. After the addition was complete, the solution was allowed to warm to room temperature and the pH was found to be 5. A 2-ml portion of 2 N NaOH was added to bring the pH in the range of 9-10 and the solution was extracted three times with Et₂O. The aqueous phase was again cooled to 0° and sufficient 3 N HCl was added to bring the pH to 2. The oil which separated was extracted into EtOAc. The aqueous phase was saturated with NaCl and extracted twice more with EtOAc. The EtOAc phase was extracted with a saturated NaCl solution
containing a little Na$_2$CO$_3$ to remove the excess HCl. After drying over Na$_2$SO$_4$ the EtOAc was removed by rotary evaporation leaving a white frothy solid. The product was dissolved in 15 ml of EtOAc by warming. After cooling at 4$^\circ$ overnight, a 0.4 g quantity of p-bromobenzoic acid was isolated. Addition of hexane to the EtOAc solution resulted in the separation of an oil. Attempts to recrystallize the product from Et$_2$O, Et$_2$O-hexane, and MeOH-H$_2$O met with similar results. Finally, the product was dissolved in 10 ml of EtOH and 300 ml of Et$_2$O, and 8.1 ml (7.5 g, 41 mmol) of dicyclohexylamine (DCHA) was added. After cooling at 4$^\circ$ overnight, 15.9 g (81%) of Bz(Br)-Pro-DCHA was isolated. This crude product with a mp of 218-220.5$^\circ$ was recrystallized from 175 ml of EtOH to give: 8.3 g (43%); mp 220-222$^\circ$; [$\alpha$]$^24_{546}$ -55.8$^\circ$ (c 1, MeOH). Several additional crops of crystals were obtained by concentrating the filtrate, but they had low melting points and were discarded. Elemental analysis calculated for C$_{24}$H$_{35}$N$_2$O$_3$Br: C, 60.11; H, 7.37; N, 5.84; Br, 16.66. Found: C, 60.05; H, 7.30; N, 5.99; Br, 16.70.

Solid Phase Synthesis of Bz(Br)-Pro-Leu-Gly-NH$_2$. The steps which result in the attachment of one amino acid residue are shown in Table 2. For this synthesis, 3.00 g of resin which contained 0.25 mmol/g of Boc-Gly was used. A 2.5-fold molar excess of Boc-Ile or Bz(Br)-Pro was used in each coupling step. The ninhydrin test (Kaiser et al. 1970) was used after each cycle to determine the completeness of the reaction. In both couplings one repetition was sufficient for complete reaction.
Table 2. Shortened sequence used in solid phase synthesis.

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Purpose</th>
<th>Solvent or reagent</th>
<th>Vol. (ml)</th>
<th>Time (min)</th>
<th>Repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>wash</td>
<td>$\text{CH}_2\text{Cl}_2$</td>
<td>30</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>deprotect</td>
<td>TFA-$\text{CH}_2\text{Cl}_2$-anisole (25:24:1)</td>
<td>30</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>wash</td>
<td>$\text{CH}_2\text{Cl}_2$</td>
<td>30</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>neutralize</td>
<td>DIEA-$\text{CH}_2\text{Cl}_2$ (1:9)</td>
<td>30</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>wash</td>
<td>$\text{CH}_2\text{Cl}_2$</td>
<td>30</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>couple</td>
<td>Boc-amino acid in $\text{CH}_2\text{Cl}_2$</td>
<td>10</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>flush</td>
<td>$\text{CH}_2\text{Cl}_2$</td>
<td>5</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>couple</td>
<td>DCC-$\text{CH}_2\text{Cl}_2$</td>
<td>10</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>flush</td>
<td>$\text{CH}_2\text{Cl}_2$</td>
<td>5</td>
<td>90</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>wash</td>
<td>$\text{CH}_2\text{Cl}_2$</td>
<td>30</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>wash</td>
<td>EtOH</td>
<td>30</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
Before the Bz(Br)-Pro-DCHA could be used it had to be converted from the salt to the free carboxylic acid. For this synthesis, 1.0 g of the salt was suspended in 50 ml of Et₂O, and 10% aqueous citric acid solution was added until two clear phases remained. The phases were separated and the aqueous phase extracted with two 50-ml portions of EtOAc. The combined organic phases were extracted with H₂O, dried over Na₂SO₄, and the solvents were removed by rotary evaporation. The foamy semi-solid residue was dissolved in 10 ml of CH₂Cl₂ and used in the synthesis.

Removal of the Peptide from the Resin and Purification. After completion of the two coupling cycles, the peptide-resin was suspended in 50 ml of anhydrous MeOH saturated at 0° with NH₃. The vessel was sealed and placed in a desiccator over CaSO₄ where it was stirred by a magnetic stirrer for 3 days at room temperature. The sealed vessel was cooled to 0°, the seal was removed, and the NH₃ and MeOH were removed by rotary evaporation. The resin was extracted with MeOH and the product was crystallized from MeOH-H₂O: wt 302 mg (86%); mp 230-232°; [α]$_{546}^{24}$ -122.1° (c 0.5, MeOH). Elemental analysis calculated for C$_{20}$H$_{27}$N$_4$O$_4$Br: C, 51.39; H, 5.83; N, 11.99; Br, 17.10. Found: C, 51.34; H, 5.90; N, 12.05; Br, 17.19.

Conclusion

Excellent individual crystals of this derivative could be obtained by dissolving 250 mp in 8 ml of MeOH (hot) and adding H₂O until the solution was turbid. Preliminary studies showed them to be
orthorhombic and work is presently under way toward solving the X-ray structure.

**Synthesis of Cys(pBrBzl)-Pro-Leu-Gly-NH$_2$**

The desirability of being able to extend the study of the conformation of the tripeptide side chain of oxytocin by the next amino acid residue is obvious. A great deal about the conformation at the juncture of the side chain and the ring and the effects of the ring on the side chain conformation could be learned by comparing the conformation of the tetrapeptide with the conformations proposed for the hormone. It could be determined whether cis-trans isomerism occurs in the Cys-Pro peptide bond of the tetrapeptide while in the solid state as it occurs in solution (Hruby, Brewster, and Glasel 1971).

Both Z-Cys(Bzl)-Pro-Leu-Gly-NH$_2$ (Bodanszky and du Vigneaud 1959, p. 2504) and Cys(Bzl)-Pro-Leu-Gly-NH$_2$ (Ressler and du Vigneaud 1954) can be obtained in crystalline forms which would appear to be suitable for X-ray structure determination. At the time this project was begun, great difficulty was being encountered in refining the data obtained from Cys(Bzl)-Pro-Leu-Gly-NH$_2$. The nature of the problem was not understood and could have been caused by several factors such as disorder in the crystal or large thermal motions. The introduction of a heavy atom would be most desirable to use as a handle for solving the X-ray structure. The HBr salt of the tetrapeptide was prepared, but it could not be crystallized. In order to deviate as little as possible from Cys(Bzl)-Pro-Leu-Gly-NH$_2$, it was decided to introduce a bromine
atom on the benzyl ring of the S-benzyl protecting group of the cysteine residue.

**Experimental Section**

Cys(pBrBzl)-Pro-Leu-Gly-NH₂ was synthesized by preparing S-p-bromobenzylcysteine, Z-Cys(pBrBzl), synthesizing Z-Cys(pBrBzl)-Pro-Leu-Gly-NH₂ by the solid phase method, and removing the Z group to give the product.

**Synthesis of S-p-Bromobenzylcysteine.** This compound was prepared in the same manner as S-benzylcysteine (du Vigneaud, Audrieth, and Loring 1930). A solution of Na (0.75 g, 0.12 mol) was prepared in 250 ml of anhydrous liquid NH₃ and cystine (6.0 g, 0.025 mol) was added slowly. After the addition was complete, the blue color was maintained for 10 min. The excess Na was reacted with a just sufficient amount of NH₄Cl, and p-bromobenzyl bromide (12.5 g, 0.05 mol) was added. The solution was stirred for 60 min while being cooled with a dry ice-acetone bath. The NH₃ was removed by evaporation and lyophilization. The product was dissolved in 150 ml of H₂O, but it precipitated after standing for 10 min. The pH was adjusted to 4 with HOAc and the slurry was cooled to 4°. The precipitate was collected, washed with H₂O and Et₂O, and dried in vacuo: wt 16.5 g; mp 199-201°. This crude product was recrystallized from EtOH-H₂O (1:1): wt 9.15 g (63%); mp 206.5-207°; [α]$_{D}^{25}$ + 3.3° (c 0.5, DMSO).

**Synthesis of Z-Cys(pBrBzl).** This product was prepared in the same manner as Z-Cys(Bzl) (Harrington and Mead 1936). A solution of S-p-bromobenzylcysteine (3.00 g, 10.4 mmol) was prepared in 5.2 ml of
2 N NaOH. The solution was cooled to 0° and treated with benzylxycarbonyl chloride (2.3 g, 13 mmol) and 5.2 ml of 2 N NaOH. Both were added dropwise over a 30-min period. The product separated as an oil which had a greater density than the aqueous phase. The reaction mixture was extracted with Et₂O, made acidic to pH 2 with 5 N HCl, and saturated with NaCl. The product was extracted into EtOAc which was then dried with Na₂SO₄. The EtOAc was removed by rotary evaporation and the oily residue was dissolved in warm benzene. No precipitation occurred upon cooling the solution unless several drops of H₂O were added. In this manner 2.8 g (64%) of Z-Cys(βBrBzl) was isolated. The product gave a single spot on tlc on Silica Gel-G in the system CHCl₃-HOAc (95:5). The melting point of this product varied considerably depending upon the solvent of crystallization. Crystallization from wet EtOAc-hexane gave large blocks of crystals (mp 71-74°) while recrystallization of already crystalline material from anhydrous EtOAc-hexane gave small needles (mp 136-138°). Any attempt to dry in vacuo any of the crystalline forms regardless of how they were obtained resulted in a heavy oil which resolidified upon addition of H₂O or after standing in moist air. The sample for optical rotation was easily recrystallized from EtOH-H₂O: [α]₂⁰²⁴°₅₄₆°=-52.8° (c 1, MeOH).

Solid Phase Synthesis of Z-Cys(βBrBzl)-Pro-Leu-Gly-NH₂. This product was synthesized in the same manner as Z-Cys(Bzl)-Pro-Leu-Gly-NH₂ (Hruby and Barstow 1972). The steps shown on Table 2 and the procedure described for the synthesis of Bz(Br)-Pro-Leu-Gly-NH₂ were used (see page 19). The peptide-resin was treated as before with NH₃-MeOH.
(see pages 19 and 21) and the product was purified by recrystallization from MeOH-H₂O: wt 0.76 g (99%); mp 185-187°; [α]²⁴₅₄₆°-80.5° (c 1, MeOH).

The product gave a single spot on tlc on Silica Gel-G in the system 1-BuOH-HOAc-H₂O-pyridine (15:3:12:10).

**Removal of the Benzoxycarbonyl Group.** A solution of Z-Cys-(pBrBzl)-Pro-Leu-Gly-NH₂ (203 mg, 0.27 mmol) was prepared in 60 ml of 2.7 N-HBr-HOAc and stirred for 60 min. The HBr salt was precipitated by the addition of 450 ml of anhydrous Et₂O, collected, washed with Et₂O, and dried in vacuo. The salt was dissolved in 1 ml of MeOH and passed through a short column of Rexyn 201 (OH⁻ cycle). The product was eluted with MeOH which was then removed by rotary evaporation. The product was triturated to a solid with Et₂O. It was reprecipitated from MeOH with Et₂O: wt 80 mg (48%); mp 68-70°.

**Conclusion**

Before much work could be done to obtain Cys(pBrBzl)-Pro-Leu-Gly-NH₂ in a crystalline form suitable for X-ray studies, the X-ray structure of Cys(Bzl)-Pro-Leu-Gly-NH₂ and its isomorphous seleno-analog were reported (Rudko, Lovell, and Low 1971). At about the same time there appeared the nmr study of Pro-Leu-Gly-NH₂, Z-Pro-Leu-Gly-NH₂, and Cys(Bzl)-Pro-Leu-Gly-NH₂ (Hruby, Brewster, and Glasel 1971). The nmr work demonstrated the existence of cis-trans isomerism about the Cys-Pro bond in a ratio of 2:3 in DMSO solution. The sharpness of the peaks and the absence of an observable temperature effect indicated that the barrier of interchange was very high. Furthermore, the nmr work suggested a conformation which placed the sulfur atom near the -NH₂ of
the glycinamide. The structure determined by X-ray analysis (Rudko et al. 1971) showed the peptide to have the trans conformation about all the peptide bonds with a particular head-to-tail interaction involving a hydrogen bond present.

As mentioned before, great difficulty was encountered in trying to refine the data for Cys(Bzl)-Pro-Leu-Gly-NH₂ obtained by X-ray diffraction. One problem was the appearance of weak OkO reflections present for odd values of k. These reflections varied in intensity from crystal to crystal and were inconsistent with the space group P2₁ determined for the compound. They were attributed to a disorder phenomenon by Rudko and coworkers who did not discuss it further. Certainly one type of disorder could have been caused by the concurrent crystallization of the cis isomer.
A few neurohypophysial hormone analogs in which the N-terminal amino group has been replaced by H have been obtained in crystalline forms. One example is deamino-oxytocin (Ferrier et al. 1965), the analog of oxytocin in which the Cys\textsuperscript{1} residue has been replaced by β-mercaptocaptopropionic acid (β-MPA). The biological activities of deamino-oxytocin were found to be greater than oxytocin: rat uterus, 795 vs. 450 units/mg and avian vasodepressor (AVD), 965 vs. 450 units/mg. Despite the fact that deamino-oxytocin is crystalline, work on its X-ray crystal structure has not appeared. It was thought, therefore, that some progress might be made by reducing the size of the problem. This could be accomplished by synthesizing the ring portion of deamino-oxytocin by itself. As mentioned before, deamino-pressinamide, the ring portion of deamino-vasopressin with the C-terminal ending in a carboxamide group, has been prepared in a crystalline form (Ferger et al. 1972). It was thus hoped that synthetic deaminotocinoic acid and deaminotocinamide might be obtained in a crystalline form. These compounds were also of potential interest for their pharmacological activities and for studies of peptide conformations in solution.
Synthesis of Deaminotocinamide and Deaminotocinoic Acid

Deaminotocinamide, $\beta$-MPA-Tyr-Ile-Gln-Asn-Cys-NH$_2$, and deaminotocinoic acid, $\beta$-MPA-Tyr-Ile-Gln-Asn-Cys, were prepared in the manner described in the literature (Hruby, Ferger, and du Vigneaud 1971; Hruby, Smith, Linn et al. 1972). The protected hexapeptide, $\beta$-MPA(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-ONB, was prepared in the stepwise manner as used in a synthesis of oxytocin (Bodanszky and du Vigneaud 1959, p. 5688) beginning from $p$-nitrobenzyl-S-benzylcysteinate $p$-toluenesulfonate and using suitably protected amino acid $p$-nitrophenyl esters. The synthesis of deaminotocinamide was carried out by first refluxing the protected hexapeptide in liquid NH$_3$ to convert the $p$-nitrobenzyl ester to the carboxamide and then by removing the protecting S-benzyl groups by adding Na metal to the NH$_3$ solution. The residue remaining after evaporation of the NH$_3$ was dissolved in 0.1% HOAc, the pH was adjusted to 8.5 with 3 N NH$_3$, and the disulfide bond was formed by oxidation with 0.01 M $K_3$Fe(CN)$_6$. The synthesis of deaminotocinoic acid was performed in an identical manner with exception that the hexapeptide was introduced into a refluxing solution of Na in liquid NH$_3$. The $p$-nitrobenzyl ester was, therefore, removed by reduction leaving the free C-terminal acid (see Appendix B for further details).

Crystallization Attempts on Deaminotocinamide and Deaminotocinoic Acid

Two attempts were made to obtain crystalline deaminotocinamide. In the first, 16 mg of deaminotocinamide was dissolved in warm H$_2$O and allowed to cool. Only a small amount of yellow fuzz separated after
several weeks. The precipitate was filtered off and the filtrate was lyophilized to recover the deaminotocinamide. In the second attempt 0.2 N HOAc was used as the solvent, but results were similar to the first attempt. Though deaminotocinamide may still be obtained in a crystalline form it is perhaps not surprising that it has not crystallized under conditions giving crystalline deamino-oxytocin since there is no reason to suppose it should have the same conformation. Indeed, it has been shown (Brewster, Glasel, and Hruby 1972) that deaminotocinamide must have a different average conformation in solution than the ring portion of deamino-oxytocin.

The attempt to obtain crystalline deaminotocinoic acid from 0.1 N HOAc met with some success. From 16 mg, 0.77 mg of crystalline material was obtained, but it was not suitable for X-ray diffraction studies. A recent report (Muhlemann et al. 1972) indicated that deaminotocinoic acid was obtained in crystalline form using the same technique as described above.

**Pharmacological Activities of the Tocin Ring Compounds**

At this point the pharmacological activities demonstrated by the polypeptides synthesized began to be of as great an interest as the conformation studies. It was found that the deamino analogs of the tocin rings possessed about ten times more oxytocic activity than the tocin rings. Thus deaminotocinoic acid had 3.7 units/mg while tocinoic acid had only 0.2-0.3 units/mg of oxytocic activity on rat uteri (Hruby, Smith, Linn et al. 1972). Deaminotocinamide had .34.2 units/mg
while tocinamide had 3.2 units/mg (Hruby, Ferger, and du Vigneaud 1971). Clearly the rings of oxytocin and deamino-oxytocin possess intrinsic oxytocic activity. On the other hand, the rings are not sufficient for avian vasodepressor activity.

It was at this time that Pro-Leu-Gly-NH$_2$ was reported to be the melanophore stimulating hormone release inhibiting factor (MSH-RIF or MRIF) in rats (Celis et al. 1971) and frogs (Nair, Castin, and Schally 1971). Pro-Leu-Gly-NH$_2$ failed to show MRIF activity in the assay system used here (Bower and Hadley 1972; Bower, Hadley, and Hruby 1971). This prompted a look into possible MRIF activity for the ring compounds. Tocinoic acid and tocinamide were found to have potent MRIF activities in the rat, hamster, bull frog (Rana catesbeiana), and toad (Bufo marinus) but not in the frog (Rana pipiens) (Bower et al. 1971; Hruby, Smith, Bower, and Hadley 1972). The MRIF activity of the deamino analogs has not been investigated at this time. For a copy of the published synthetic and pharmacological work on the tocin ring compounds, see Appendix B.
CHAPTER 4

THE RING MOIETY OF ISOTOCIN AND GLUMITOCIN

Isotocin ([Ser$^4$, Ile$^8$]-oxytocin) and glumitocin ([Ser$^4$, Gln$^8$]-oxytocin) are found in the neurohypophyses of teleost fishes and elasmobranchs, respectively (Acher et al. 1962, Acher et al. 1965). Evidence indicates that they influence adenohypophysial activity and control osmoregulation in these fishes (Morel and Jard 1968). Until the recent isolation of aspertocin ([Asn$^4$]-oxytocin) (Acher et al. 1972), isotocin and glumitocin represented the only hormones in which a difference in the ring moiety from oxytocin or the vasopressins was present. The two hormones have a serine residue in place of glutamine at position 4 of the tocin ring. Because of the biological activities found in the tocin ring compounds, it became of great interest to synthesize the corresponding ring compounds of isotocin and glumitocin in order to see what intrinsic activities they might possess. Any crystallinity discovered would be an added bonus.

Synthesis Using Precursors with C-Terminal Nitrobenzyl Ester Protection

Good success was obtained in the synthesis of the tocin ring compounds using nitrobenzyl ester C-terminal protection. Also, the availability of the option of either forming the carboxamide group or the free acid from the same precursor encouraged the use of a similar scheme for the synthesis of the corresponding [Ser$^4$]-tocin compounds.
In the synthesis of the tocin ring compounds, benzyloxy carbonyl protection was used on all amino acid residues except glutamine. However, since the removal of the benzyloxy carbonyl group required conditions which also removed O-benzyl protecting groups from hydroxyl functions and since three more coupling steps are required after the attachment of the O-benzyl protected serine residue, it was decided to use Boc protection after the attachment of the serine residue. The Boc group was removed under conditions which allowed the benzyl ethers to remain intact.

Experimental Section

The protected peptides were prepared in the stepwise manner (Bodanszky and du Vigneaud 1959, p. 5688) using either nitrophenyl esters (Bodanszky 1955) or N-hydroxysuccinimide esters (Anderson, Zimmerman, and Callahan 1964).

**Synthesis of Boc-Ser(Bz1)-Asn-Cys(Bz1)-ONB.** A solution of Z-Asn-Cys(Bz1)-ONB (Hruby, Ferger, and du Vigneaud 1971) (2.08 g, 3.5 mmol) was prepared in 23 ml of anhydrous HOAc and 21 ml of 5.3 N HBr-HOAc. After stirring for 60 min, the resulting HBr salt was precipitated by the addition of 300 ml of anhydrous Et$_2$O. The salt was collected, washed with Et$_2$O, and dried in vacuo. After drying, the salt was dissolved in 14 ml of DMF and neutralized to pH 7 (Fisher Indicator Solution) with N-methylmorpholine. The solution was then treated with Boc-Ser(Bz1)-ONSu (1.6 g, 4.0 mmol) for 48 hr. The product was precipitated with H$_2$O, collected, washed with H$_2$O and H$_2$O-EtOH (1:1), and dried in vacuo: wt 2.33 g (90%); mp 163-164.5°; $[\alpha]_D^{22}$ -15.4°
Synthesis of Boc-Ile-Ser(Bz1)-Asn-Cys(Bz1)-ONB. A solution of Boc-Ser(Bz1)-Asn-Cys(Bz1)-ONB (2.1 g, 2.85 mmol) was prepared in 26 ml of trifluoroacetic acid (TFA) containing 3 ml of anisole. After stirring for 25 min the resulting TFA salt was precipitated by the addition of 300 ml of anhydrous Et$_2$O. The salt was collected, washed with Et$_2$O, and dried in vacuo. The dried salt was dissolved in 15 ml of DMF and treated as previously described with N-methylmorpholine and Boc-Ile-ONSu (1.05 g, 3.2 mmol). The product was precipitated, washed and dried in vacuo as before: wt 2.17 g (90%); mp 181.5-182.5 °C; [α]$_D^{22}$-16.2° (c 0.5, DMF). Elemental analysis calculated for C$_{42}$H$_{54}$N$_6$O$_{11}$S: C, 59.27; H, 6.41; N, 9.88. Found: C, 59.09; H, 6.25; N, 9.90.

Synthesis of Boc-Tyr(Bz1)-Ile-Ser(Bz1)-Asn-Cys(Bz1)-ONB. This compound was prepared as described above from Boc-Ile-Ser(Bz1)-Asn-Cys(Bz1)-ONB (1.87 g, 2.2 mmol) and Boc-Tyr(Bz1)-ONp (1.1 g, 2.4 mmol). The product was precipitated with 2 ml of H$_2$O and 70 ml of EtOAc, collected, and washed with EtOAc, EtOH, EtOH-H$_2$O (1:1), EtOH, and Et$_2$O. The product was dried in vacuo: wt 1.73 g (71%); mp 221-220 °C; [α]$_D^{22}$-10.0° (c 0.5, DMF). Elemental analysis calculated for C$_{58}$H$_{69}$N$_7$O$_{13}$S: C, 63.07; H, 6.31; N, 8.88. Found: C, 62.97; H, 6.21; N, 8.81.

Synthesis of Z-Cys(Bz1)-Tyr(Bz1)-Ile-Ser(Bz1)-Asn-Cys(Bz1)-ONB. This compound was prepared as described previously from Boc-Tyr(Bz1)-Ile-Ser(Bz1)-Asn-Cys(Bz1)-ONB (724 mg, 0.65 mmol) and Z-Cys(Bz1)-ONp
(0.4 g, 0.9 mmol). The product was precipitated, washed, and dried as above: wt 810 mg (94%); mp 245-246.5°; \([\alpha]_{D}^{22} = -22.0° (c 0.5, \text{DMF}).\) Elemental analysis calculated for C_{71}H_{78}N_{8}O_{14}S_{2}: C, 64.03; H, 5.92; N, 8.42. Found: C, 63.82; H, 5.80; N, 8.43.

**Synthesis of [Ser^4]-Tocinoic Acid While Attempting to Synthesize [Ser^4]-Tocinamide.** Into 250 ml of anhydrous liquid NH₃ was placed Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB (200 mg, 0.15 mmol). Unlike the glutamine containing analog used to prepare tocinamide (Hruby, Ferger, and du Vigneaud 1971), this protected hexapeptide was not soluble in NH₃. After refluxing for two hours with stirring, the hexapeptide remained undissolved. The suspension was treated with Na for 3 min, after which the excess Na was reacted with HOAc. The NH₃ was removed by evaporation and lyophilization. The slightly yellow powder was dissolved in 500 ml of deaerated 0.1% aqueous HOAc. The pH was adjusted to 8.5 with 3 N NH₃, and 31 ml of 0.01 M K₃Fe(CN)₆ was added slowly. After 30 min of stirring the pH was adjusted to 5 with 6 N HOAc, and 5 ml of Rexyn 203 (Cl⁻ cycle) was added. After 15 min the resin was filtered off and washed with 0.2 N HOAc. The combined filtrates were lyophilized to dryness. The residue was dissolved in 7 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH-EtOH-pyridine-HOAc-H₂O (4:1:1:0.4:6.4) used for the purification of tocinamide and tocinoic acid (Hruby, Ferger, and du Vigneaud 1971; Hruby, Smith, Linn et al. 1972) and subjected to partition chromatography (Yamashiro 1964; Yamashiro, Gillessen, and du Vigneaud 1966) on a column of Sephadex G-25 (100-200 mesh) 2.9 x 60 cm. The peptide material
was detected by the Folin-Lowry method (Lowry et al. 1951) and the portion comprising the major peak at \( R_f \) 0.25 was isolated by evaporation and lyophilization: wt 70.0 mg. This product was dissolved in 4 ml of 0.2 N HOAc and further purified by gel filtration (Porath and Flodin 1959) on a column of Sephadex G-25 (200-270 mesh) 2.9 x 64 cm equilibrated with 0.2 N HOAc. The product emerged as a large peak at 90% of the column volume flanked by two small peaks. The main peak was isolated by lyophilization: wt 31.3 mg. The gel filtration was carried out a second time. The product emerged as a single peak and was isolated by lyophilization: wt 30.4 mg (29%). The peptide gave one large and one very small, faster moving spot (estimated at less than 5% of the product) on TLC using Silica Gel-G in the system 1-BuOH-HOAc-H\(_2\)O-pyridine (15:3:12:10). The impurity was confirmed to be \([\text{Ser}^4]\)-tocinamide by TLC comparison with \([\text{Ser}^4]\)-tocinamide prepared by two methods discussed later. Amino acid analysis (Spackman, Stein, and Moore 1958) of the major product after 36-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 1.0; Cys, 1.8; Ile, 1.0; Tyr, 0.80; NH\(_3\), 1.3. Elemental analysis calculated for \(\text{C}_{28}\text{H}_{41}\text{N}_{10}\text{O}_{2}\cdot\text{H}_{2}\text{O}\): C, 46.84; H, 6.05; N, 13.66. Found: C, 46.73; H, 6.28; N, 13.63.

**Synthesis of \([\text{Ser}^4]\)-Tocinoic Acid.** A solution of Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB (210 mg, 0.16 mmol) was prepared in 24 ml of 2.6 N HBr-HOAc and stirred for 60 min. The salt, HBr-Cys-(Bzl)-Tyr-Ile-Ser-Asn-Cys(Bzl)-ONB, was precipitated with anhydrous Et\(_2\)O, collected, washed with Et\(_2\)O, and dried in vacuo. The salt was added to a solution of Na in 100 ml of liquid NH\(_3\) and the blue color...
was allowed to persist for 3 min after which the excess Na was reacted with HOAc. The residue remaining after the removal of the NH₃ by evaporation and lyophilization was treated as described previously with 46 ml of K₃Fe(CN)₆. The crude yellowish product was subjected to gel filtration in 50% aqueous HOAc on a column of Sephadex G-15 (40-120 μ beads) 2.2 x 111 cm. The product emerged at 51% of the column volume preceded by a large polymer peak at 34-41% of the column volume. Both peaks were isolated by lyophilization. The polymer peak contained 94 mg and the product weighed 24 mg. The polymer was treated again with Na-NH₃ and K₃Fe(CN)₆. The crude product isolated plus the 24 mg obtained from the 50% HOAc gel filtration were subjected to partition chromatography in the same system as described before. The peptide which emerged as a large peak at Rₓ 0.22 was isolated by evaporation and lyophilization: wt 48 mg. This product was further purified by gel filtration on Sephadex G-25 in 0.2 N HOAc as described before. The product emerged as a large peak at 90% of the column volume flanked by two smaller peaks. The main peak was isolated by lyophilization: wt 18 mg (11%). This product gave a single spot on tlc identical to the spot comprising the major component obtained in the first attempt to synthesize [Ser⁴]-tocinamide.

Synthesis of [Ser⁴]-Tocinamide. To remove all the protecting groups except the S-benzyl and p-nitrobenzyl groups, Z-Cys(Bzl)-Tyr-(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB (269 mg, 0.2 mmol) was treated as previously described with HBr-HOAc. The resulting HBr salt was introduced into 300 ml of anhydrous NH₃ where it dissolved immediately.
After refluxing for 2 hr the solution was treated with Na and the resulting sulfhydryl compound was treated as described previously with \( \text{K}_3\text{Fe(CN)}_6 \). The crude product was subjected to partition chromatography in the previously described system on a column of Sephadex G-25 (100-200 mesh) 2.2 x 60 cm. The product emerged as a large peak at \( R_f \) 0.30 preceded by a small polymer peak and was isolated by lyophilization: wt 88.6 mg. This product was further purified by gel filtration on a column of Sephadex G-25 (200-270 mesh) 2.9 x 64 cm in 0.2 N HOAc. The product emerged at 87% of the column volume as a single large peak which had a significant forepart and tail. The center of the peak was isolated by lyophilization. It weighed 56 mg and was a white powder under vacuum, but it acquired a yellowish tinge when exposed to air. This product was further subjected to gel filtration on a column of Sephadex G-15 (40-120 \( \mu \) beads) 2.2 x 113 cm in 50% aqueous HOAc. The product emerged as a large peak at 49% of the column volume flanked by two small peaks. The material in the large peak was isolated by lyophilization: wt 30.0 mg (21%). The product gave a single spot on tlc on Silica Gel-G in the system \( \text{l-BuOH-HOAc-H}_2\text{O-pyridine (15:3:12:10)} \) corresponding to the faster moving impurity present in the first attempt to synthesize this compound in which the major product was \( [\text{Ser}^4] \)-tocinoic acid. Amino acid analysis after 34-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 1.0; Cys, 2.0; Ile, 1.1; Tyr, 0.81; NH\textsubscript{3}, 2.0. Elemental analysis calculated for \( \text{C}_{28}\text{H}_{42}\text{N}_8\text{O}_9\text{S}_2 \): C, 48.12; H, 6.07; N, 16.04. Found: C, 48.13; H, 5.91; N, 16.03.
Synthesis of β-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB. This compound was prepared from Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB (1.50 g, 1.35 mmol) by condensation with β-MPA(Bzl)-ONp (0.50 g, 1.57 mmol). The product weighed 1.03 g (65%) and had a mp of 233.5-235.5°.

First Attempt to Synthesize [Ser^4]-Deaminotocinamide. A portion of β-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB (300 mg, 0.254 mmol) was first treated with HBr-HOAc in hopes that the removal of the O-benzyl groups from tyrosine and serine would improve its solubility in liquid NH₃. Unfortunately the product of this treatment, β-MPA(Bzl)-Tyr-Ile-Ser-Asn-Cys(Bzl)-ONB, was not a salt since there is no N-terminal group on the molecule as in the case of [Ser^4]-tocinamide and it was isolated as an extremely hygroscopic, fine, and difficult to filter solid: wt 131 mg (52%). This product was dissolved in liquid NH₃ and was refluxed for 2 hr. The solution was treated as before with Na and the product with K₃Fe(CN)₆. When an attempt was made to purify the crude product in the system used for deaminotocinamide (Hruby, Ferger, and du Vigneaud 1971), 1-BuOH-C₆H₆-H₂O (2:1:3), no peptide material was eluted from the column. A significant amount of crude product, thought at the time to be polymers, was insoluble in the partitioning system.

Synthesis of β-MPA(Bzl)-Tyr-Ile-Ser-Asn-Cys(Bzl)-ONB. Because of the trouble encountered when β-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB was treated with HBr-HOAc to prepare this compound, it was decided to prepare it by other means. Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB (1.03 g, 0.933 mmol) was treated for 60 min in 30 ml of
The product, HBr–Tyr–Ile–Ser–Asn–Cys(Bzl)–ONB, was precipitated with Et₂O, collected, washed with Et₂O, and dried in vacuo. The salt was then condensed in the previously described manner with β-MPA(Bzl)–ONp (0.35 g, 1.1 mmol). The product weighed 0.56 g (60%) and had a mp of 221–223°.

Second Attempt to Synthesize [Ser⁴]–Deaminotocinamide. A 250-mg portion of β-MPA(Bzl)–Tyr–Ile–Ser–Asn–Cys(Bzl)–ONB was refluxed in liquid NH₃ for several hours. This was followed by treatment with Na and K₃Fe(CN)₆ in the usual manner. When the crude product was subjected to partition chromatography as in the first attempt to synthesize it, the results were nearly identical. A small amount (7.3 mg) of bright yellow powder was eluted and much material remained insoluble in the solvent system.

Synthesis of [Ser⁴]–Deaminotocinoic Acid. A portion of β-MPA-(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB (301 mg, 0.254 mmol) was treated with Na in NH₃ followed by K₃Fe(CN)₆ in the usual manner. The crude product, which had a dark brown color at this point, was dissolved in 7 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH-EtOH-pyridine-HOAc-H₂O (5:1:1:0.15:7) which had been used successfully in the purification of deaminotocinoic acid (Hruby, Smith, Linn et al. 1972). It was subjected to partition chromatography on a column of Sephadex G-25 (100-200 mesh) 2.9 x 61 cm. The product emerged at an Rₜ of 0.42 preceded by a huge polymer peak which overlapped the product slightly. The 56.3 mg of tan powder isolated by lyophilization was subjected to a second partition chromatography in the same system.
but on a different column of Sephadex G-25 (100-200 mesh). This column was 2.2 x 60 cm. The 35.0 mg of white powder obtained by lyophilization was further purified by gel filtration on a column of Sephadex G-15 (40-120 μ beads) 2.2 x 113 cm in 50% aqueous HOAc. The product emerged as a single peak at 56% of the column volume and was isolated by lyophilization: wt 12.5 mg (4%). The product gave a single spot on tlc on Silica Gel-G in the system 1-BuOH-HOAc-H₂O-pyridine (15:3:12:10). Amino acid analysis following 36-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 0.90; Ile, 1.0; Tyr, 0.91; NH₃, 1.2; Cys, 0.48; mixed disulfide of Cys and β-MPA, 0.59. Elemental analysis calculated for C₂₈H₄₀N₆O₁₀S₂: C, 49.10; H, 5.90; N, 12.27. Found: C, 49.03; H, 5.92; N, 12.11.

Discussion of Results and Problems Encountered

While the scheme to synthesize the [Ser⁴]-tocin compounds using the C-terminal nitrobenzyl ester precursors was so successful in the synthesis of the tocin compounds, the scheme was of only limited success here. First, the fully protected hexapeptide, Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-ONB, was found to be too insoluble in liquid NH₃ for its complete conversion into the corresponding C-terminal carboxamide. When this procedure was tried, only a trace of [Ser⁴]-tocinamide was produced with the major product being [Ser⁴]-tocinoic acid resulting from the reductive cleavage of the nitrobenzyl ester by Na. Although this solubility problem could be eliminated by prior treatment of the protected peptide with HBr-HOAc to remove the
N-terminal and O-benzyl protecting groups to synthesize \([\text{Ser}^4]\)-tocinamide, the same procedure could not be applied successfully to \(\beta\text{-MPA(BzI)-Tyr(BzI)-Ile-Ser(BzI)-Asn-Cys(BzI)-ONB}\) to synthesize \([\text{Ser}^4]\)-deaminotocinamide. Even when the more soluble hexapeptide, \(\beta\text{-MPA(BzI)-Tyr-Ile-Ser-Asn-Cys(BzI)-ONB}\), was prepared in another manner, no success was obtained in the synthesis of \([\text{Ser}^4]\)-deaminotocinamide. It should, however, be pointed out that \([\text{Ser}^4]\)-deaminotocinamide may indeed have been prepared, but that it was found later to be almost totally insoluble in the \(1\text{-BuOH-}C_6H_5-H_2O\) (2:1:3) partitioning system which was being used in attempting to purify it.

Another problem with this scheme was the very low yields of purified peptide obtained. Yields from the protected hexapeptides ranging from a high of 21% for \([\text{Ser}^4]\)-tocinamide to a low of 4% for \([\text{Ser}^4]\)-deaminotocinoic acid were obtained. Some of the losses were due to the third problem of this scheme, the formation of troublesome yellow by-products from the reduction of the nitro-moiety. These colored impurities often necessitated the use of additional purification steps to rid the products of them.

**Attempted Synthesis Using the Solid Phase Technique**

The rather poor results obtained by the classical solution techniques using the C-terminal nitrobenzyl esters prompted an investigation into the feasibility of using the solid phase technique.

Amino acid residues such as asparagine and glutamine, which normally give problems when attempts are made to attach them to a
resin by the refluxing EtOH method (Gutte and Merrifield 1971), have been successfully attached under the mild conditions of shaking in DMF with 0.9 equivalents of Et₃N (Marglin 1971). Since Boc-Cys(Bzl) also presents problems, it was decided to use this method to attach Boc-Cys(MeOBzl) to a resin.

Resin substituted with Boc-Cys(MeOBzl) to the extent of 0.6 mmol/g (aldimine test) was prepared by shaking 2.00 g of chloromethylated resin (2.07 meq/g) and Boc-Cys(MeOBzl) (0.69 g, 2.0 mmol) with Et₃N (0.25 ml, 1.8 mmol) in 12 ml of DMF for 120 hr. Using this resin, an attempt was made to synthesize β-MPA(Bzl)-Tyr-Ile-Ser-Asn-Cys-(MeOBzl). Standard solid phase techniques were used (see Table 2). The asparagine residue was attached using the nitrophenyl ester, Boc-Asn-ONp, in DMF. Serine and tyrosine were protected on the hydroxyl function by benzyl ethers. The finished peptide was removed from the resin by treatment with HBr-TFA (Stewart and Young 1969, p. 40). The product was purified by reprecipitation from DMF with EtOH and Et₂O. There was obtained a 34% yield of material with a mp of 166-180°C. At least three major components were shown to be present by tlc.

There are at least two likely reasons for the appearance of more than one product in tlc. First, if the S protection were partially lost from the cysteine residues, at least four components could be present. More could appear by the air oxidation of the -SH group to a disulfide bond. A follow-up treatment of Cys(MeOBzl) with HBr-TFA showed the p-methoxybenzyl group to be cleaved to the extent of 98%.

Another way in which multiple products could be formed would be if
cysteine became attached to the resin via the sulfur atom. This could lead to a host of cyclic products.

**Synthesis Using Precursors with C-Terminal Benzyl Ester Protection**

The problems encountered with the precursors having C-terminal nitrobenzyl esters and the mixed products obtained by the solid phase method made it necessary to work out a third scheme to synthesize the [Ser\(^4\)]-tocin compounds. It should be recalled that one of the problems with the C-terminal nitrobenzyl protected peptides was the formation of highly colored side products from the reduction of the nitro group. This problem could be eliminated by using C-terminal benzyl protection. The nitrobenzyl group was used in the synthesis of the tocin compounds because benzyleoxycarbonyl N-terminal protection was used requiring the use of HBr-HOAc for its removal. The nitrobenzyl ester group was stable to HBr-HOAc where the benzyl ester group was partially susceptible to cleavage (Schwarz and Arakawa 1959). However, in the synthesis of [Ser\(^4\)]-tocin compounds it was desirable to keep the O-benzyl protection on the serine residue by the use of N-terminal Boc protection because of its milder removal conditions. It would be a simple matter to extend the Boc protection to the asparagine residue, thus eliminating the need for the more tenacious nitrobenzyl ester in favor of the benzyl ester which was employed in the successful synthesis of the pressin ring compounds (Ferger et al. 1972).

Another problem encountered in the first scheme was the failure of refluxing liquid NH\(_3\) to convert the nitrobenzyl ester to the
carboxamide of the fully protected hexapeptides without prior treatment to remove the protecting groups. To avoid this problem it was proposed to convert half of the pentapeptide, Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl, to the corresponding carboxamide by treating it in MeOH saturated with NH₃. Thus [Ser⁴]-tocinoic acid and [Ser⁴]-deamino-tocinoic acid would be prepared from the hexapeptides obtained by condensing the pentapeptide benzyl ester with Z-Cys(Bzl)-ONp or β-MPA(Bzl)-ONp, respectively. The corresponding C-terminal carboxamide hexapeptides, [Ser⁴]-tocinamide and [Ser⁴]-deaminotocinamide, would be prepared by condensing Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH₂ with either Boc-Cys(Bzl)-ONSu or β-MPA(Bzl)-ONp.

Experimental Section

Synthesis of Boc-Ser(Bzl)-Asn-Cys(Bzl)-OBzl. A solution of Boc-Asn-Cys(Bzl)-OBzl (Ferger et al. 1972) (5.65 g, 11 mmol) was prepared in 20 ml of TFA containing 2 ml of anisole. After stirring for 30 min at room temperature the TFA and anisole were removed by rotary evaporation at 1 mm Hg pressure and 35°. The oily residue was triturated with Et₂O, evaporated to dryness and triturated again under Et₂O until it solidified. The solid was collected, washed with Et₂O, and dried in vacuo. The TFA salt was dissolved in 15 ml of DMF and neutralized to pH 7 with N-methylmorpholine. The solution was treated with Boc-Ser(Bzl)-ONSu (5.0 g, 12.4 mmol) for 40 hr. The product was precipitated by the addition of 100 ml of H₂O, collected, washed with H₂O, EtOAc, and Et₂O, and dried in vacuo: wt 5.94 g (78%); mp 144-145°; [α]²⁵⁴₄₆ -22.7° (c 0.5, DMSO). Elemental analysis calculated for
Synthesis of Boc-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl. A solution of Boc-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (3.22 g, 4.65 mmol) in 15 ml of TFA and 1.5 ml of anisole was stirred 30 min. The TFA salt was precipitated with 300 ml of anhydrous Et₂O, collected, washed with Et₂O, and dried in vacuo. This TFA salt was condensed as described previously with Boc-Ile-ONSu (1.70 g, 5.2 mmol): wt 3.34 g (92%); mp 197-199°C; [α]$_{546}^{25}$ -25.1° (c 0.5, DMSO). Elemental analysis calculated for C$_{42}$H$_{55}$N$_{5}$O$_{8}$S: C, 62.58; H, 6.89; N, 8.69. Found: C, 62.47; H, 6.95; N, 8.70.

Synthesis of Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl. This peptide was prepared from Boc-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (5.6 g, 6.8 mmol) and Boc-Tyr(Bzl)-0NSu (3.44 g, 7.5 mmol) in the manner described previously: wt 6.51 g (90%); mp 219-220°C; [α]$_{546}^{25}$ -15.1° (c 0.5, DMSO). Elemental analysis calculated for C$_{58}$H$_{70}$N$_{11}$O$_{11}$S: C, 65.75; H, 6.67; N, 7.93. Found: C, 65.75; H, 6.60; N, 7.97.

Synthesis of Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl. This peptide was prepared by first treating Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (0.95 g, 0.90 mmol) with TFA as described before and then by condensing with Z-Cys(Bzl)-ONp for 20 hr. An additional equivalent of N-methylmorpholine was added and the reaction was continued for 6 hr longer. The product was precipitated with EtOAc-H₂O (30:1). The precipitate was collected, washed with EtOH, EtOH-H₂O (1:1), EtOH, Et₂O, and dried in vacuo: wt 0.94 g (81%); mp 238-240.5°C; [α]$_{546}^{25}$ -26.1°
(c 0.5, DMSO). Elemental analysis calculated for $C_{71}H_{79}N_{12}O_{12}S_{2}$: C, 66.27; H, 6.20; N, 7.62. Found: C, 66.37; H, 6.22; N, 7.75.

Synthesis of [Ser$^4$]-Tocinoic Acid. A 200 mg (0.15 mmol) portion of Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl was treated with 16 ml of 2.7 N HBr-HOAc for 50 min. The resulting HBr salt was precipitated with anhydrous Et$_2$O, collected, washed with Et$_2$O, and dried in vacuo. The salt was added to a refluxing solution of Na dissolved in anhydrous liquid NH$_3$. The solution was stirred and the blue color allowed to persist for 4 min. The excess Na was reacted with HOAc and the NH$_3$ was removed by evaporation and lyophilization. The residue was dissolved in 400 ml of deaerated 0.1% aqueous HOAc. The pH was adjusted to 8.5 with 3 N NH$_3$ and the sulfhydryl compound was oxidized with 30 ml of 0.01 M K$_3$Fe(CN)$_6$. After 15 min the pH was adjusted to 4 with HOAc, and 4 ml of Rexyn 203 (Cl$^-$ cycle) was added. The suspension was stirred for 15 min, after which the resin was filtered off and washed with 10% aqueous HOAc. To the combined filtrates 40 ml of re-distilled 1-BuOH was added to prevent bumping when the total volume was reduced to 150 ml by rotary evaporation. The remaining solution was lyophilized to dryness. The residue was dissolved in 5 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH-HOAc-$H_2$O (4:1:4) and subjected to partition chromatography (Yamashiro 1964, Yamashiro et al. 1966) on a 2.9 x 61 cm column of Sephadex G-25 (100-200 mesh). The peptide material was detected by reading the absorbance of the eluate at 280 nm, and the part comprising the major peak at $R_f$ 0.21 was isolated by evaporation and lyophilization: wt 53.0 mg. This product was
dissolved in 4 ml of 20% aqueous HOAc and further purified by gel filtration (Porath and Flodin 1959) on a 2.9 x 65 cm column of Sephadex G-25 (200-270 mesh) equilibrated with 20% aqueous HOAc. The compound emerged, preceded by a small impurity, as a sharp peak at 75% of the column volume and was isolated by lyophilization: wt 30.1 mg (29%); $[\alpha]^{25}_{546} +34.5^\circ$ (c 0.5, DMSO). The peptide gave a single spot on tlc on Silica Gel-G in the system 1-BuOH-HOAc-H$_2$O-pyridine (15:3:12:10) identical to the [Ser$^4$]-tocinoic acid prepared before (see pages 35-36).

**Synthesis of $\beta$-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl.**

This compound was prepared from Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (1.0 g, 0.94 mmol) and $\beta$-MPA(Bzl)-ONp (0.32 g, 1.0 mmol) in the same manner as described for Z-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl: wt 0.85 g (79%); mp 242-244°; $[\alpha]^{25}_{546} -15.9^\circ$ (c 0.5, DMSO). Elemental analysis calculated for C$_{63}$H$_{72}$N$_6$O$_{10}$S$_2$: C, 66.52; H, 6.39; N, 7.40. Found: C, 66.58; H, 6.30; N, 7.42.

**Synthesis of [Ser$^4$]-Deaminotocinoic Acid.** A 220 mg (0.185 mmol) portion of $\beta$-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl which had been ground to a very fine powder was treated in a solution of excess Na in liquid NH$_3$ for 10 min. The excess Na was reacted with HOAc and the NH$_3$ was evaporated and lyophilized to dryness. The sulfhydryl compound was dissolved in a solution containing 220 ml of de-ionized, deaerated H$_2$O and 205 ml of deaerated acetone. To the solution was added 54.5 mg (0.191 mmol) of CH$_2$ICH$_2$I (twice recrystallized from Et$_2$O dissolved in 15 ml of acetone. The progress of the oxidation was followed by the Ellman test (Ellman 1959) and was judged
complete after 15 min. To the solution 5 ml of HOAc was added, the acetone was removed by rotary evaporation, and the aqueous solution was lyophilized to dryness. The residue was dissolved in 6 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH-EtOH-pyridine-HOAc-H_2O (5:1:1:0.15:7) and subjected to partition chromatography on a 2.9 x 64 cm column of Sephadex G-25 (100-200 mesh). The peptide was detected by the Folin-Lowry method (Lowry et al. 1951) and the part comprising the major peak at R_f 0.41 was isolated by evaporation and lyophilization: wt 93.6 mg. This product was dissolved in 8 ml of HOAc-H_2O (4:1) and further purified by gel filtration on a 2.9 x 64 cm column of Sephadex G-25 (100-200 mesh) equilibrated with 50% aqueous HOAc. The compound emerged as a single sharp peak at 67% of the column volume and was isolated by lyophilization: wt 67.8 mg (54%); [α]_25^{25} 546 -50.1° (c 0.5, DMSO). The compound gave a single spot on tlc on Silica Gel-G in the system 1-BuOH-HOAc-H_2O-pyridine (15:3:12:10) identical to the compound prepared on pages 39-40.

**Synthesis of Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH_2.** A suspension of finely ground Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl (1.13 g, 1.07 mmol) was prepared in 100 ml of anhydrous MeOH saturated with NH_3 at 0°. The reaction vessel was sealed and placed in a desiccator at room temperature. The suspension was stirred with a magnetic stirrer for three days. The MeOH and NH_3 were removed by rotary evaporation. The residue was triturated twice with MeOH which was removed by rotary evaporation. The product was triturated with Et_2O, collected, and washed with Et_2O. After drying in vacuo, it was dissolved in DMF.
and precipitated with H$_2$O, collected, washed with H$_2$O and Et$_2$O, and dried in vacuo: wt 0.85 g (86%); mp 245-248° (dec.); [$\alpha$]$^\text{D}_{546}$ -19.5° (c 0.5, DMSO). Elemental analysis calculated for C$_{51}$H$_{65}$N$_7$O$_{10}$S•H$_2$O: C, 62.10; H, 6.86; N, 9.94. Found: C, 62.48; H, 6.68; N, 9.86.

**Synthesis of Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH$_2$.** This peptide was prepared from Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH$_2$ (0.85 g, 0.86 mmol) and Boc-Cys(Bzl)-ONSu as described previously. The product was precipitated with H$_2$O, collected and washed with H$_2$O, EtOH, and Et$_2$O. After drying in vacuo the product weighed 1.00 g (97%); mp 260-262°; [$\alpha$]$^\text{D}_{546}$ -33.6° (c 0.5, DMSO). Elemental analysis calculated for C$_{61}$H$_{76}$N$_{11}$S$_2$: C, 63.07; H, 6.61; N, 9.65. Found: C, 63.07; H, 6.59; N, 9.70.

**Synthesis of [Ser$^4$]-Tocinamide.** The Boc group of Boc-Cys(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH$_2$ (243 mg, 0.209 mmol) was removed by treatment with TFA as previously described. The TFA salt obtained was treated with Na-NH$_3$ and the disulfide bond was formed by oxidation with K$_3$Fe(CN)$_6$ as described for [Ser$^4$]-tocinoic acid (see page 46). The crude product was dissolved in 6 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH-EtOH-pyridine-HOAc-H$_2$O (4:1:1:0.4:6.4) and subjected to partition chromatography on a 2.9 x 60 cm column of Sephadex G-25 (100-200 mesh). The peptide material was detected by the Folin-Lowry method and the part comprising the major peak at R$_f$ 0.33 was isolated by rotary evaporation and lyophilization: wt 101 mg. This product was dissolved in 4 ml of 50% aqueous HOAc and further purified by gel filtration on a 2.2 x 111 cm column of Sephadex G-15.
(40-120 μ beads) equilibrated with 50% aqueous HOAc. The peptide, preceded by a small impurity, emerged at 45% of the column volume: wt 67.8 mg (48%); $[\alpha]_{546}^{25} = -75.5^\circ$ (c 0.5, DMSO). The peptide gave a single spot on tlc on Silica Gel-G in the system 1-BuOH-HOAc-$H_2$O-pyridine (15:3:12:10) identical to the material prepared on pages 36-37.

**Synthesis of β-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH$_2$.** This peptide was prepared from Boc-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH$_2$ (0.65 g, 0.66 mmol) and β-MPA(Bzl)-ONp (0.24 g, 0.75 mmol) in the manner described for the preparation of β-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-OBzl: wt 0.57 g (81%); mp 262-263$^\circ$ (dec.); $[\alpha]_{546}^{25} = -25.5^\circ$ (c 0.5, DMSO). Elemental analysis calculated for $C_{56}H_{67}N_7O_9S_2\cdotH_2O$: C, 63.18; H, 6.55; N, 9.21. Found: C, 63.06; H, 6.32; N, 9.60.

**Synthesis of [Ser$^4$]-Deaminotocinamide.** A 225 mg (0.21 mmol) portion of finely ground β-MPA(Bzl)-Tyr(Bzl)-Ile-Ser(Bzl)-Asn-Cys(Bzl)-NH$_2$ was treated as described for the synthesis of [Ser$^4$]-deaminotocinoic acid (see pages 39-40). An attempt was made to dissolve the crude product in 8 ml of the upper phase and 4 ml of the lower phase of the system 1-BuOH-$C_6H_6$-$H_2$O (2:1:3). A large quantity of undissolved material was filtered off and saved. The filtrate was subjected to partition chromatography on a 2.9 x 60 cm column of Sephadex G-25 (100-200 mesh). No peptide material could be detected emerging from the column. The insoluble material was dissolved in 5 ml of HOAc-$H_2$O (4:1) and subjected to gel filtration on a 2.2 x 111 cm column of Sephadex G-15 (40-120 μ beads). The peptide material was detected by reading the absorbance of the eluate at 280 nm. The product emerged, preceded by a
small peak, at 57% of the column volume and was isolated by lyophilization: wt 256 mg. This crude product, containing much NaOAc, was precipitated from 30 ml of 30% aqueous HOAc and subjected to a second gel filtration as described above: wt 29.8 mg (21%); $[\alpha]_{546}^{25}$ -96.5° (c 0.5, DMSO). The peptide gave a single spot on tlc on Silica Gel-G in the system 1-BuOH-HOAc-H$_2$O-pyridine (15:3:12:10). Amino acid analysis following 36-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Asp, 1.1; Ser, 1.0; Ile, 0.97; Tyr, 0.79; Cys, 0.51; mixed disulfide of cysteine and β-MPA, 0.62. Elemental analysis calculated for C$_{28}$H$_{41}$N$_7$O$_9$S$_2$: C, 49.17; H, 6.05; N, 14.34. Found: C, 48.99; H, 6.08; N, 14.15.

Results and Discussion of the Synthesis

All four [Ser$^4$]-tocin ring compounds were obtained in moderate to good yields in highly purified form using this last scheme. For the synthesis of [Ser$^4$]-tocinoic acid it was found that the prior removal of the Z and O-Bzl groups from the protected hexapeptide allowed a smoother reaction with the Na-NH$_3$ over a shorter time. Of course the Boc group of the protected hexapeptide used in the synthesis of [Ser$^4$]-tocinamide had to be removed prior to the Na-NH$_3$ treatment since this group would not otherwise be cleaved. The hexapeptides used for the synthesis of the deamino analogs required fine grinding and longer contact with the Na-NH$_3$ for complete removal of the protecting groups.
Comparison of Synthetic Schemes

For the purposes of this comparison scheme I will be the procedure using C-terminal p-nitrobenzyl ester protection and scheme II will be the procedure using C-terminal benzyl ester protection. In order to compare the two schemes it is necessary to look at the results obtained for each compound. Using scheme I an 11% yield of pure $[\text{Ser}^4]^{-}$tocinoic acid was obtained. In order to obtain even this low yield it was necessary to gel filter the crude product and recycle the polymer peak. Using scheme II a 29% yield of pure $[\text{Ser}^4]^{-}$tocinoic acid was obtained. Scheme II required no special recycling and the highly purified product was easily obtained by one partition chromatography and one gel filtration. Something should be said about the different purification techniques used in the two schemes. In scheme I the partition system was the same one used for tocinoic acid, $1\text{-BuOH-EtOH-pyridine-HOAc-H}_2\text{O}$ (4:1:1:0.4:6.4). At best $[\text{Ser}^4]^{-}$tocinoic acid was just barely soluble in this system and some product was certainly lost due to this insolvability. In scheme II a newly developed solvent system, $1\text{-BuOH-HOAc-H}_2\text{O}$ (4:1:4), was employed in which $[\text{Ser}^4]^{-}$tocinoic acid was easily soluble. A similar situation existed with the gel filtration solvents. During the execution of scheme I, the final gel filtration step was carried out in 0.2 N HOAc. It was later found that $[\text{Ser}^4]^{-}$tocinoic acid was only sparingly soluble in 0.2 N HOAc. Gel filtrations were run in 20% aqueous HOAc during scheme II.

Both methods were utilized to prepare $[\text{Ser}^4]^{-}$tocinamide. Scheme I gave a 21% yield of purified product while scheme II gave a 48% yield.
The difference in yields was primarily due to the ease of purification of the crude product. The execution of scheme I led to the formation of highly colored side products which necessitated additional purification steps than those required in scheme II.

The formation of colored side products was also a serious problem in the synthesis of \([\text{Ser}]^4\)-deaminotocinoic acid by scheme I. The crude product required two partition chromatography steps and two gel filtrations to give a colorless product and only a 47% yield. Execution of scheme II gave a 54% yield, requiring only one partitioning and one gel filtration.

It should be recalled that no \([\text{Ser}]^4\)-deaminotocinamide was isolated using scheme I. The fault here surely must lie with the purification procedure. During scheme I the crude product was being subjected to partition chromatography in the system \(1\text{-BuOH-C}_6\text{H}_6\text{-H}_2\text{O (2:1:3)}\). It was later found that the product was virtually insoluble in that system. In order to be soluble, \([\text{Ser}]^4\)-deaminotocinamide required a high percentage of HOAc in any system. The problem was that when sufficient HOAc was added to make the product soluble, the distribution coefficient became so large that the product eluted too quickly. Using a combination of gel filtration and precipitation \([\text{Ser}]^4\)-deaminotocinamide was finally purified.

In general, it may be said that scheme II was far superior to scheme I. However, it should be pointed out that some of the
improvement seen in scheme II was due to the additional experience gained from performing scheme I.

Some Properties of [Ser$^4$]-Tocin Compounds

The properties of the compounds discussed here include some of their physical properties and some of their biological activities.

Physical Properties

The marked difference in solubilities of the [Ser$^4$]-tocin compounds as compared to the tocin compounds led to many of the problems encountered during the synthesis of the [Ser$^4$]-tocin compounds. All of the [Ser$^4$]-tocin compounds were much less soluble than their tocin counterparts in aqueous and non-aqueous solvents. While the tocin compounds were easily soluble in 0.2 N HOAc, the solutions of the [Ser$^4$]-tocin compounds needed to study their biological activities had to be prepared from HOAc-H$_2$O solutions. An example of how these solutions were prepared is illustrated by the preparation of a 0.05 mg/ml solution of [Ser$^4$]-deaminotocinamide, the least soluble of all the [Ser$^4$]-tocin compounds.

Solution of [Ser$^4$]-Deaminotocinamide. A 0.54-mg portion of [Ser$^4$]-deaminotocinamide was dissolved by warming in 0.200 ml of HOAc and 0.100 ml of H$_2$O and diluting to 10 ml with warm H$_2$O. No precipitation occurred after 5 days. The pH was adjusted to 6-6.5 with 4 N NaOH. No precipitation occurred after 7 days longer.
**Crystallinity of [Ser$^4$]-Tocin Compounds.** All but [Ser$^4$]-tocinamide have been obtained in crystalline form from HOAc-H$_2$O solutions. The crystalline form of [Ser$^4$]-tocinoic acid was obtained by dissolving 13.5 mg in 0.5 ml of H$_2$O and 0.05 ml of HOAc. Fine needles grew when the solution was cooled. Larger crystals could be obtained by slow evaporation of more dilute solutions: mp 193-195° (dec.). Attempts to grow still larger crystals required for X-ray diffraction are still under way.

Thin sheets of crystalline [Ser$^4$]-deaminotocinoic acid were obtained by cooling a boiling solution of 19.6 mg in 0.25 ml of HOAc and 0.50 ml of H$_2$O: mp 232-234° (dec.).

Fine granules of crystalline [Ser$^4$]-deaminotocinamide were obtained by cooling a boiling solution of 17.8 mg in 0.45 ml of HOAc and 0.50 ml of H$_2$O: mp 245-246° (dec.).

**Pharmacological Activities**

All four compounds were checked for oxytocic activity (Holton 1948, Munsick 1960), avian vasodepressor activity [Coon 1939; The Pharmacopeia of the United States of America (hereafter referred to as U.S. Pharmacopeia) 1970, p. 469], and rat pressor activity (U.S. Pharmacopeia 1970, p. 771). None of them showed any of these activities at the highest dose levels permitted by their solubilities. All four compounds except [Ser$^4$]-tocinoic acid were also tested as inhibitors of the response of oxytocin in all three assay systems. The only measurable inhibition was exhibited in the oxytocic assay by [Ser$^4$]-tocinamide and [Ser$^4$]-deaminotocinamide, which were about 1/30 as strong as
deamino-penicillamineoxytocin (Schulz and du Vigneaud 1966) in this respect. In preliminary experiments [Ser$^4$]-tocinoic acid and [Ser$^4$]-tocinamide did not show any MRIF activity (Hruby, Smith, Bower, and Hadley 1972). The two compounds were also tested for milk ejecting activity in mouse mammary tissue in vitro using a procedure similar to that used by van Dongen and Hays (1966). At $10^{-6}$ g/ml, [Ser$^4$]-tocinoic acid was found to have measurable milk ejecting activity. Deamino-oxytocin was used as a standard and was active at $10^{-10}$ g/ml. With the differences in molecular weight, this makes deamino-oxytocin 14,000 times more effective than [Ser$^4$]-tocinoic acid. Similarly [Ser$^4$]-tocinamide, active at $5 \times 10^{-7}$ g/ml, was 7,000 times less effective than deamino-oxytocin.
CHAPTER 5

MODIFICATIONS OF SOLID PHASE PEPTIDE SYNTHESIS

One of the goals of any synthetic chemist is the improvement of the techniques in use. In this section are described three innovations which were attempted in hopes of improving the solid phase method. The first technique was the synthesis of a cysteine derivative with an improved S protecting group. The second innovation was the use of a macroreticular resin for the solid phase support. The last innovation was the attempt to attach cysteine to a solid phase resin by a thioether linkage.

The 3,4-Dimethylbenzyl Group as a Protecting Group of Cysteine

For most syntheses done by solid phase, N-Boc protection is employed with the final removal of the side chain protecting groups accomplished by treatment with liquid HF. There are, therefore, four necessary requirements for the side chain protecting groups: (1) they must repeatedly survive the conditions used to remove the Boc groups, (2) they must be efficiently removed by HF, (3) they should give rise to no side products during the synthesis, and (4) the amino acid derivatives must couple efficiently to the peptide resin. Currently the p-methoxybenzyl group is used to protect the thiol group of cysteine instead of the benzyl group because of the latter group's inefficient
cleavage in HF. It has been shown, however, that the \( p \)-methoxybenzyl group is cleaved to the extent of 27% by treatment with 50% TFA in \( \text{CH}_2\text{Cl}_2 \) for 23 hr (Yamashiro, Noble, and Li 1972). These authors found that the 3,4-dimethylbenzyl group under the same conditions was cleaved to the extent of only 0.2% yet was completely removed by liquid HF. The investigators synthesized the C-terminal cyclic dodecapeptide human growth hormone, Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Phe, with this derivative. At this time no method for the preparation of this derivative has appeared. Here is reported the synthesis of Cys(3,4-Me\(_2\)Bzl) and Boc-Cys(3,4-Me\(_2\)Bzl) carried out in our laboratory for use in the solid phase synthesis of neurohypophysial polypeptides.

**Experimental Section**

The compound S-(3,4-dimethylbenzyl)-cysteine was prepared in the same manner as S-benzylcysteine (duVigneaud et al. 1930). The Boc group was attached by the pH-stat method (Schnabel 1967) and Boc-Cys-(3,4-Me\(_2\)Bzl) was isolated as the DCHA salt.

**Synthesis of S-(3,4-Dimethylbenzyl)-cysteine.** A solution of Na (9.2 g, 0.4 mol) in 350 ml of anhydrous liquid NH\(_4\) was prepared and cystine (24 g, 0.1 mol) was added to the NH\(_4\) in small portions while the solution was constantly stirred with a glass stir-bar and a magnetic stirrer. Additional small bits of Na were required after all of the cystine had been added in order to maintain the blue color for 10 min. The excess Na was reacted with solid NH\(_4\)Cl and a paddle stirrer was introduced into the solution. Chloromethyl-3,4-dimethylbenzene (34 g, 31 ml, 0.22 mol) which had been freshly redistilled (106-107.5\(^\circ\)
at 21 torr) was added dropwise over a 20-min period. The slurry was stirred an additional 30 min after which the remaining NH₃ was lyophilized to dryness. The residue was dissolved in 200 ml of H₂O and filtered to remove a small amount of insoluble material. The aqueous filtrate was extracted twice with 100-ml portions of Et₂O. With the mechanical stirrer attached, the pH of the solution was adjusted to 4 with HOAc. In order to maintain a slurry, 400 ml of H₂O were required. The precipitate was collected, washed with H₂O and Et₂O, and dried in vacuo: wt 37.5 g (78%), mp 174-177° sintering from 165°. The product was taken up in 2 l. of boiling 50% aqueous EtOH and filtered while hot to remove 1.8 g of unreacted cystine. The filtrate was concentrated by evaporation to 1.5 l. then cooled overnight at 4°. The precipitate was collected, washed with H₂O and Et₂O, and dried in vacuo: wt 31.1 g, mp 184-186° (lit. 195-197°). A second crop was obtained which weighed 2.8 g and melted at 180-182°. The total yield was 33.9 g (71%).

Synthesis of Boc-Cys(3,4-Me₂Bzl)-DCHA. A slurry of S-(3,4-dimethylbenzyl)-cysteine (12.0 g, 0.05 mol) was prepared in 10 ml of dioxane, 10 ml of H₂O, and 10 ml of 4 N NaOH. The pH of the solution was 10.0. To the mixture 8.0 ml of t-butylazidoformate (0.055 mol) was added and the pH was maintained at 9.8 by addition of 4 N NaOH for 10 hr. The solution was extracted with Et₂O. A layer of EtOAc was placed over the aqueous phase and the pH was adjusted to 3-4 with solid citric acid. The aqueous phase was saturated with NaCl and the phases were separated. The aqueous phase was extracted twice more with EtOAc. The combined EtOAc extractions were back extracted with H₂O containing a
little 1 N NaHCO₃ until the pH of the aqueous phase was about 7. The EtOAc phase was dried with Na₂SO₄ and the EtOAc was removed by rotary evaporation. The oily residue was taken up in Et₂O and cooled. A small amount of flocculent material separated. The precipitate was collected, washed with Et₂O and dried in vacuo: wt 0.3 g. The nmr of this precipitate suggested that it was Boc-Cys(3,4-Me₂Bzl)-O-3,4-Me₂Bzl. The ether was removed from the filtrate by rotary evaporation. Several attempts were made to crystallize small portions of the product to use as seed crystals. The following systems were tried: MeOH-H₂O, Et₂O-hexane, and EtOAc-H₂O-hexane. The product remained as an oil. The oil was dissolved in 100 ml of EtOH and 9.9 ml of dicyclohexylamine was added. After standing overnight the DCHA salt was precipitated by the dropwise addition of 100 ml of H₂O. The product separated as needles. It was collected, washed with H₂O and hexane, and dried in vacuo: wt 23.2 g (89%), mp 118-120° (lit. 122-124°), [α]D²⁴ +4.1° (c 1, MeOH); lit. [α]D²⁴ -20.0° (c 2.3, 80% HOAc). The product showed a trace of slower moving impurity on tlc on Silica Gel-G in the systems amylOH-pyridine-H₂O (7:7:5) and BuOH-HOAc-H₂O (4:1:5). However, after the product was freed from the DCHA salt with citric acid, it gave a single, uniform, ninhydrin negative spot using the same two solvent systems. Elemental analysis calculated for C₂₉H₄₈N₂O₄S: C, 66.87; H, 9.31; N, 5.38. Found: C, 66.61; H, 9.32; N, 5.55. This compound is currently being used in the solid phase synthesis of some neurohypophysial hormones.
Work with Macroreticular Resin

During the synthesis of polypeptides of moderate size it has been observed that as the chain length increased the effective concentration of the N-terminal -NH$_2$ group decreased (Ehler 1972). When hydrolysis of the peptide resin was performed, however, the results suggested that while some of the peptide was being lost from the resin much of the peptide was still attached, but it was unavailable for further reaction. One explanation for this phenomenon might be seen by a closer look at the resin.

The first amino acid was attached by the nucleophilic substitution of chlorine as benzyl chloride by the carboxylate of the Boc-amino acid. Undoubtedly some of the chloromethyl groups were located in sites which were to some degree sterically hindered. As the peptide became larger, these sites became more sterically hindered until a point was reached where reagents could not penetrate to the N-terminal residue and chain growth stopped.

A solution to this problem might be found in the use of a macroreticular resin with its large pore size. The size of the pore would not restrict the growth of the polypeptide. This idea could be carried one step further by introducing the chloromethyl groups on the resin in a solvent which does not swell the resin to any great extent. In this manner the chloromethyl groups would presumably be located more at the surface rather than buried deep in the resin. For this purpose Amberlite XE-305 (Rohm and Haas), a low cross-linked macroreticular copolymer of styrene-divinylbenzene, was chosen. The resin had a
surface area of 48 m$^2$/g, a pore size distribution ranging from 600-2000 Å with an average pore diameter of 1400 Å, and came in beads of 20-50 mesh. Excellent results have been reported using a similar resin in the synthesis of tetra- and hexapeptides by the stepwise procedure and by fragmentation condensation (Sano, Tokunaga, and Kun 1971). Here are reported procedures for obtaining various degrees of chloromethylation and attachment of the first amino acid as well as the synthesis of Z-Cys(Bzl)-Tyr(Bzl)-Ile-Leu-Asn-Cys(MeOBzl)-Pro-Arg(TOS)-Gly-NH$_2$, the protected nonapeptide of [Leu$^4$]-vasotocin.

Experimental Section

Preparation of Chloromethyl Resin (Merrifield 1964). In each preparation 10 g of Amberlite XE-305 was suspended in 40 ml of solvent for 10 min to allow the wetting of the resin. To this was added 40 ml of chlorodimethyl ether and a measured quantity of SnCl$_4$ catalyst. After the reaction the resin was collected, washed with 200 ml of dioxane-H$_2$O (3:1), 200 ml of dioxane-3 N HCl (3:1), dioxane, H$_2$O, MeOH, and dried in vacuo. The amount of chlorine was determined by the Volhard method (Stewart and Young 1969, pp. 55-56). In one case an elemental analysis for chlorine was also done to check the accuracy of the procedure. Results were the same within experimental error. The amount of chlorine introduced onto the resin using various conditions is shown on Table 3.
Table 3. Degree of chloromethylation

<table>
<thead>
<tr>
<th>Solvent</th>
<th>SnCl₂ (ml)</th>
<th>Time (min)</th>
<th>Temp</th>
<th>Cl (meq/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃</td>
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<td>30</td>
<td>reflux</td>
<td>4.2</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>2</td>
<td>10</td>
<td>ambient</td>
<td>2.3</td>
</tr>
<tr>
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<td>2</td>
<td>20</td>
<td>0°</td>
<td>1.9</td>
</tr>
<tr>
<td>CHCl₃</td>
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</tr>
<tr>
<td>hexane</td>
<td>2</td>
<td>15</td>
<td>0°</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Preparation of Boc-Gly-OResin (Stewart and Young 1969, p. 32).

Two of the chloromethylated resins prepared above were used to make Boc-Gly-OResin. For the first preparation 5.00 g of the 0.49 meq/g resin was suspended in 25 ml of EtOH with Boc-Gly (1.05 g, 6.0 mmol) and Et₃N (0.75 ml, 0.54 g, 5.4 mmol). The mixture was refluxed for 72 hr. The resin was filtered off, washed with EtOH, H₂O, CH₂Cl₂, and dried in vacuo. A small portion was treated with TFA and the amount of glycine attached was determined by the aldimine test (Esko et al. 1968; Ehler 1972, pp. 39-41). A substitution of 0.148 mmol/g was found.

For the second preparation 5.00 g of the 1.2 meq/g resin, which had been prepared in hexane, was suspended in 25 ml of EtOH with Boc-Gly (2.62 g, 15 mmol) and Et₃N (1.35 g, 1.9 ml, 13.5 mmol). The mixture was refluxed for 72 hr and worked up as described above. The substitution, as determined by the aldimine test, had the following values: 0.50, 0.54, 0.65, 0.67, and 0.75 mmol/g. A value determined by amino acid analysis was 0.87 mmol/g.
Synthesis of Z-Cys(Bzl)-Tyr(Bzl)-Ile-Leu-Asn-Cys(MeOBzl)-Pro-Arg(TOS)-Gly-NH$_2$. For this synthesis 2.50 g of 0.148 mmol/g Boc-Gly-OResin was used. The sequence of the steps of a typical coupling cycle is shown on Table 2. The asparagine residue was coupled as Boc-Asn-ONp in an overnight reaction which was continued for 2 hr after an equivalent of DIEA was added. All the other residues were coupled with DCC in 60 min reactions. An ninhydrin test (Kaiser et al. 1970), after step 1 of the sequence, was performed for each cycle and showed greater than 99.4% coupling. Due to a calculation error an 8-fold excess of Boc-amino acid and DCC was used instead of the usual 2.5-fold excess. The peptide was removed from the resin by treatment with NH$_3$ in MeOH as previously described (see page 19). The product was extracted from the resin with DMF and precipitated with H$_2$O. After drying in vacuo it weighed 0.35 g (61%) and melted at 241.5-242.5°. No additional product was obtained from the filtrate or by a second treatment of the resin with NH$_3$.

Discussion of the Suitability of Amberlite XE-305

A marked superiority of Amberlite XE-305 over conventional resins was not demonstrated. When the resin was chloromethylated in CHCl$_3$, the availability of the chloromethyl groups did not seem to be any greater than that seen for conventional resins. Only about one-third of the chlorine available was substituted. While a greater percentage of the chlorine could be substituted using the hexane prepared resin, there is no evidence that similar results could not be obtained with
standard resins. The apparent non-homogeneity of the substitution was also troublesome. The synthesis of the nonapeptide went in 61% yield which was perhaps a little less than would be expected from any solid phase synthesis. While complete coupling was obtained using only 60 min reaction times, this may be attributed to the accidental 8-fold excess of reagents used as well as to the characteristics of the resin. Finally, the Amberlite XE-305 showed a distressing lack of mechanical stability. It broke up badly during the course of the synthesis.

More work is necessary using smaller bead sizes and several syntheses should be done before any real statement about its suitability as a solid phase support can be made.

**Attachment of Cysteine to a Resin by a Thioether Linkage**

The problems encountered when Boc-Cys(MeOBzl) was attached to a solid phase resin in the usual manner have been discussed (see pages 41-42). Therefore, a new method of cysteine attachment was sought. One possible solution was the attachment of cysteine to the resin by its sulfhydryl group. In this manner any competition between the carboxylate ion and the thioether present in Boc-Cys(MeOBzl) would be eliminated by the protection of the carboxyl group, leaving only the nucleophilic sulfide group to react with the benzyl chloride of the resin. The scheme proposed to accomplish this involves the formation of dimethyl bis-t-butyloxycarbonylcystinate, (Boc-Cys-OMe)₂, its treatment with Na-NH₃ to give the reduced compound, Boc-Cys-OMe, and the
reaction of the reduced compound with the chloromethylated resin to give Boc-Cys(Resin)-OMe.

This scheme offers great flexibility to any synthesis. First of all the Boc and OMe groups are not removed by the Na-NH₃ treatment necessary for the formation of the sulfide group. The Boc group of course is the preferred group for N-terminal protection in solid phase synthesis. The OMe group provides protection for the C-terminal end, but it can be removed by mild basic hydrolysis to give the free carboxylate or by ammonolysis to give the C-terminal carboxamide.

Experimental Section

Dimethyl bis-butoxycarbonylcystinate was prepared and converted to the reduced form. The reduced compound was treated with chloromethylated resins having various amounts of chlorine under several reaction conditions and in several solvents. Detection of cysteine attachment was accomplished by the ninhydrin test (Kaiser et al. 1970) after a prior treatment with TFA. When any attachment was detected, it was quantized by the aldimine test (Eska et al. 1968, Ehler 1972) or by elemental analysis for sulfur. Success in removal of the cysteine from the resin was measured by the Ellman test (Ellman 1959).

Synthesis of (Boc-Cys)₂ (Ferraro 1971). A solution of cysteine (12.0 g, 0.050 mol) was prepared in 50 ml of H₂O containing NaOH (4.0 g, 0.10 mol) in 25 ml of additional H₂O. The solution was cooled to 0-10° and 200 ml of DMF was added in a slow stream. Finally, 25 ml of t-butylazidoformate was added and the pH maintained at 10 overnight by the addition of 32 ml of 4 N NaOH from a pH-stat. The solution was poured into
300 ml of H₂O and extracted with two 100-ml portions of EtOAc. A layer of EtOAc was added over the aqueous phase and the pH of the cooled solution was adjusted to 3 with 2 N HCl. The phases were separated and the organic phase was washed with H₂O until the pH of the H₂O phase was 6-7. The organic phase was dried with Na₂SO₄ and reduced in volume to 50 ml. The volume was brought back up to 100 ml with EtOAc and the solution was warmed to dissolve a precipitate which had separated. To the warm solution sufficient hexane was added to cause turbidity. After cooling at 4° overnight, the product was collected, washed with hexane, and dried in vacuo: wt 19.5 g (89%); mp 144.5-145° (lit. 144.5-145°).

**Synthesis of (Boc-Cys-OMe)₂.** A solution of (Boc-Cys)₂ (6.38 g, 14.5 mmol) was prepared in 40 ml of anhydrous MeOH and cooled to -10°. To this was added DCC (6.7 g, 32.5 mmol) dissolved in 7 ml of MeOH. The mixture was stirred 60 min at -10°, then stored at 4° overnight. The DCU was filtered off and washed with Et₂O. The solvents of the filtrate were removed by rotary evaporation and the oily residue taken up in Et₂O. The Et₂O solution was filtered to remove more DCU and the Et₂O was removed by rotary evaporation. The treatment with Et₂O was repeated twice more until no more DCU separated from the Et₂O solution. The product was recrystallized from Et₂O solution. The product was recrystallized from Et₂O-hexane. It was collected, washed with hexane, and dried in vacuo: wt 6.0 g (87%); mp 88-91°. In the nmr spectrum the area under the peak at 3.7 ppm (corresponding to the methyl group) and the area under the peak at 1.4 ppm (corresponding to the t-butyl group) had the correct ratio of 1.0:3.0.
Attachment of Cysteine to the Resin. Measured amounts of (Boc-Cys-OMe)₂ were reduced with Na-NH₃. It was found that roughly four equivalents of Na were required before the blue color of Na persisted. An nmr of the reduced compound showed a large upfield shift of the Boc group perhaps due to the ionization of the proton of the urethane nitrogen. The reduced compound was allowed to react with different chloromethylated Amberlite XE-305 resins under a variety of conditions. The results are shown in Table 4. (For the preparation of the Amberlite resins, see pages 61-62.)

The symbol (N) in Table 4 after experiments 1, 6, and 7 was used to indicate that the reduced compound was first neutralized to pH 6 with HOAc before treatment with the resin. In addition, during experiment 7, Et₃N (8 mmol) was also added after the HOAc to establish an equilibrium between the sulfhydryl form and the sulfide ion. The pH of the final solution in experiment 7 was 6.5. In the other experiments the reduced compound was used directly as the sodium salt. In DMF these basic conditions seemed to lead to many tar-like side products, especially as the amount of Boc-Cys-OMe was increased.

Removal of Cysteine from the Resin. In order for this procedure to be of any synthetic usefulness, conditions for removal of the cysteine had to be found. Resins from several of the experiments, in which the ninhydrin and aldimine tests had indicated that cysteine had become attached, were subjected to conditions which have been used to remove the S-benzyl protecting group from cysteine (Sifferd and du Vigneaud 1935, Jost and Rudinger 1961). The results of these
<table>
<thead>
<tr>
<th>Number</th>
<th>Boc-Cys-OMe (mmol)</th>
<th>Resin g</th>
<th>meq/g Cl</th>
<th>Solvent</th>
<th>Time (hr)</th>
<th>Temp.</th>
<th>Substitution (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4 (N)</td>
<td>2.00</td>
<td>0.49</td>
<td>DMF</td>
<td>20</td>
<td>25°</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.4</td>
<td>2.00</td>
<td>0.49</td>
<td>NH₃ (l)</td>
<td>3</td>
<td>reflux</td>
<td>trace</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>1.00</td>
<td>1.9</td>
<td>DMF</td>
<td>35</td>
<td>25°</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>4.6</td>
<td>1.00</td>
<td>2.3</td>
<td>NH₃ (l)</td>
<td>7.5</td>
<td>reflux</td>
<td>0.08</td>
</tr>
<tr>
<td>5</td>
<td>4.6</td>
<td>1.00</td>
<td>2.3</td>
<td>DMF</td>
<td>48</td>
<td>25°</td>
<td>0.22</td>
</tr>
<tr>
<td>6</td>
<td>6.9 (N)</td>
<td>2.00</td>
<td>2.3</td>
<td>DMF</td>
<td>67.5</td>
<td>25°</td>
<td>trace</td>
</tr>
<tr>
<td>7</td>
<td>4.6 (N)</td>
<td>1.00</td>
<td>2.3</td>
<td>MeOH</td>
<td>20.5</td>
<td>reflux</td>
<td>0.57 (1.3)</td>
</tr>
</tbody>
</table>
experiments as determined by diluting the extraction solvent to a known volume and using the Ellman test are shown on Table 5.

Discussion of Results

These preliminary experiments have shown that the attachment of cysteine by the thioether linkage seems feasible, but its subsequent removal by the methods tried was inadequate. The failure to attach any significant amount of cysteine to the resin when liquid NH$_3$ was used as the solvent can most likely be attributed to the failure of this solvent to swell the resin. This result makes the partial successes achieved in detaching the cysteine with Na-NH$_3$ suspect. In those cases where -SH was detected after treatment of the peptide-resin with Na-NH$_3$, the reduced compound had been used directly and one might theorize that the cysteine was adhering to the resin in some fashion other than the thioester bond (see Table 5, resin number .5).

The most promising experiment in the attachment of cysteine was number 7. During the course of this experiment no colored side products were formed and the highest substitution of all the experiments was obtained. The free amine was measured by the aldimine test and found to be 0.57 mmol/g. However, when an elemental analysis for sulfur was performed, it was found to be present to the extent of 1.3 mmol/g. If all the cysteine were attached by the thioether bond, the ratio of -NH$_2$ to S should have been 1:1. An alternative mode of sulfur attachment is possible if the methoxy group were displaced by the -S$^-$ group of a second molecule of Boc-Cys-OMe.
Table 5. Removal of cysteine from the resin.

<table>
<thead>
<tr>
<th>Resin no.</th>
<th>Reagents</th>
<th>Extraction solvent</th>
<th>Percent removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>TFA, Na-NH₃</td>
<td>0.5 M HOAc; DMF-MeOH</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Na-NH₃</td>
<td>CH₂Cl₂-HOAc</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Na-NH₃</td>
<td>DMF (0.5 M in HOAc)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Na-NH₃</td>
<td>DMF-H₂O (0.5 M in HOAc)</td>
<td>53</td>
</tr>
<tr>
<td>7</td>
<td>Na-NH₃</td>
<td>MeOH (0.7 M in HOAc)</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Na-NH₃</td>
<td>DMF-H₂O (0.5 M in HOAc)</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>33% HBr-HOAc  40° 40 hr</td>
<td>DMF-H₂O (0.5 M in HOAc)</td>
<td>Trace</td>
</tr>
<tr>
<td>7</td>
<td>33% HBr-HOAc-phenol 40° 40 hr</td>
<td>DMF-H₂O (0.5 M in HOAc)</td>
<td>Trace</td>
</tr>
</tbody>
</table>
The $-\text{NH}_2$ group of this second molecule would not be detected by the aldimine test if it were involved in a facile cyclization during the removal of the Boc group by TFA.

This process would explain the fact that the sulfur was found in a ratio of approximately 2:1 over the $-\text{NH}_2$ group. Certainly there seems no reason to limit the transesterification to only two molecules and perhaps it was this process which formed the tar-like products during some experiments.
If the -OME group were too labile to prevent this transesterification, protecting the C-terminal carboxyl group as the carboxamide would solve the problem but reduce the flexibility of the scheme. (A better procedure to detach the peptide from the resin might be its treatment with liquid HF.)
CHAPTER 6

THE SYNTHESIS OF MESOTOCIN

The structure of mesotocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Ile-Gly-NH₂, differs from that of oxytocin by the substitution of an isoleucine residue for the leucine residue at position 8 in oxytocin. Mesotocin was synthesized (Jaquenoud and Boissannas 1961) three years before it was isolated from the posterior pituitary of frogs (Acher et al. 1964). It was synthesized again by a slightly different method five years later (Rudinger et al. 1969). The role of the naturally occurring peptide has not been fully investigated although it is apparent that neurohypophysial hormones are important in the osmoregulation of amphibians (Morel and Jard 1968). Since mesotocin is the naturally occurring hormone in amphibians and since MSH has such an obvious effect on these animals, it would seem very logical to investigate the MRIF activity of mesotocin and its side chain, Pro-Ile-Gly-NH₂. The ring structure is of course the tocin ring which has been discussed previously (see pages 28-30).

Attempts to Synthesize Mesotocin by Solid Phase

The protected nonapeptide of oxytocin has been prepared in high yield by the solid phase method (Bayer and Hagenmaier 1968, Manning 1968). The speed and ease with which the solid phase method was employed encouraged the use of this method for the synthesis of mesotocin.
In the first attempt Boc-Gly-OResin (4.00 g) which had been prepared from Amberlite XE-305 chloromethylated in hexane (see pages 61-63) was used. The amount of Boc-Gly attached to the resin was assumed to be 0.6 mmol/g. A 2.5-fold excess of Boc-amino acid or a 4-fold excess of Boc-amino acid nitrophenyl ester was used. A typical coupling cycle is shown on Table 2. The synthesis proceeded with 99-100% complete reactions through the attachment of the glutamine residue. The attachment of the next three amino acids required several recycles using prolonged reaction times and 1-hydroxybenzotriazole (Konig and Geiger 1970) before the completeness of the reaction was in the range of 95%. The peptide-resin was treated with MeOH-NH$_3$ as described previously (see page 19). The product was extracted from the resin with DMF, precipitated with H$_2$O, washed with H$_2$O, EtOH, Et$_2$O, and dried in vacuo: wt 0.4 g (12%); mp 238-241$^\circ$. A second crop was obtained by extracting the resin with DMSO: wt 0.35 g (11%); mp 243.5-245.5$^\circ$. The weight gain of the resin had been 2.2 g which meant that most of the product was still on the resin. Treatment of either crop of peptide with Na-NH$_3$ followed by oxidation with K$_3$Fe(CN)$_6$ and purification by partition chromatography and gel filtration gave six products. All of these products had a strong sulfhydryl odor and none of them showed any significant milk ejecting activity.

The solid phase synthesis was repeated using a standard Boc-Gly-OResin (4.00 g) containing 0.49 mmol/g of Boc-Gly. The same trouble with the coupling steps was encountered after the attachment of the glutamine residue. The weight gain of the resin was 2.21 g (97%). The
peptide-resin was again treated with MeOH-NH\textsubscript{3}. The peptide was extracted from the resin with warm DMF as before to give 0.6 g (mp 227-230°) and with DMSO to give 0.19 g (mp 244-247°). An IR of the resin revealed a strong amide carbonyl band indicating that much of the product was still attached to the resin.

The resin was treated by a second ammonolysis method which had been reported to successfully cleave peptides from solid phase supports which had not been cleaved successfully before (Parr, Yang, and Holzer 1972). The procedure involves suspending 1 g of peptide resin in 50 ml of DMF, cooling to -70°, adding 50 ml of anhydrous liquid NH\textsubscript{3}, placing the solution in a Parr Hydrogenation Apparatus, and shaking for 5 days. However, when this procedure was attempted on a scale of 5 g, the pressure in the Parr Apparatus became too great and the solution and resin were lost.

**Synthesis of Mesotocin Using Solution Techniques**

The difficulty with some of the coupling steps and the incomplete cleavage of the peptide from the solid phase support prompted the use of solution techniques for the synthesis of mesotocin. The scheme was devised so that tocinoic acid and Pro-Ile-Gly-NH\textsubscript{2} as well as mesotocin could be prepared from the same precursors. First, Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-OBzl was prepared in the stepwise manner using active esters. This protected pentapeptide was treated with HBr-TFA (Stewart and Young 1969, p. 40) to simultaneously remove all but the S-Bzl group of cysteine. The pentapeptide was then condensed with Z-Cys(Bzl)-ONp to give Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl). This
protected hexapeptide could be used to make tocinoic acid or coupled with Pro-Ile-Gly-NH$_2$ to make the protected nonapeptide of mesotocin. In the same manner as Z-Pro-Leu-Gly-NH$_2$, Z-Pro-Ile-Gly-NH$_2$ was prepared by the mixed anhydride procedure (Cash 1961).

Experimental Section

Both Boc-Asn-Cys(Bz1)-OBz1 and Boc-Gln-Asn-Cys(Bz1)-OBz1 were prepared by the stepwise active ester method (Ferger et al. 1972). The procedure was continued to prepare Boc-Ile-Gln-Asn-Cys(Bz1)-OBz1 and Boc-Tyr(Bz1)-Ile-Gln-Asn-Cys(Bz1)-OBz1 which have both been prepared by a different technique (Muhlemann et al. 1972).

Synthesis of Boc-Ile-Gln-Asn-Cys(Bz1)-OBz1. A solution of Boc-Gln-Asn-Cys(Bz1)-OBz1 (3.22 g, 5.00 mmol) was prepared in 15 ml of TFA and 1.5 ml of anisole. After stirring for 25 min, the TFA salt was precipitated by the addition of anhydrous Et$_2$O. The salt was collected, washed with Et$_2$O, and dried in vacuo. The TFA salt was dissolved in 20 ml of DMF, and N-methylmorpholine (0.56 ml, 0.505 g, 5.0 mmol) was added. The pH of the solution was 7-7.5. After the addition of Boc-Ile-ONSu (1.9 g, 5.7 mmol), the solution was stirred for 20 hr. The pH was checked and found to be 6.5, so an additional equivalent of N-methylmorpholine was added and stirring was continued for a few more hours. The product was precipitated with 60 ml of H$_2$O and 100 ml of EtOAc. The precipitate was collected, washed with EtOAc, acetone-Et$_2$O (4:1), Et$_2$O, and dried in vacuo: wt 2.20 g (58%); mp 232-232.5° (lit. 238-240°).
Synthesis of Boc-Tyr(Bz1)-Ile-Gln-Asn-Cys(Bz1)-OBz1. This peptide was prepared from Boc-Ile-Gln-Asn-Cys(Bz1)-OBz1 (2.09 g, 2.70 mmol) and Boc-Tyr(Bz1)-ONSu (1.38 g, 2.0 mmol) in the same manner as described for Boc-Ile-Gln-Asn-Cys(Bz1)-OBz1, omitting only the acetone-Et2O wash of the product: wt 2.30 g (85%); mp 242-244 ° (lit. 245-248 °); [α]24°D -20.1° (c 0.5, DMSO), lit. [α]25°D -22.6° (c 1, DMF). Elemental analysis calculated for C53H67N7O11S: C, 63.00; H, 6.70; N, 9.71. Found: C, 63.07; H, 6.90; N, 9.66.

Synthesis of Z-Cys(Bz1)-Tyr-Ile-Gln-Asn-Cys(Bz1). A solution of Boc-Tyr(Bz1)-Ile-Gln-Asn-Cys(Bz1)-OBz1 (1.21 g, 1.2 mmol) was prepared in 5 ml of TFA and 1 ml of anisole. Anhydrous HBr, which had been passed through towers of naphthalene and CaCl2, was bubbled into the solution for 50 min. Nitrogen was bubbled through the solution until vapors of HBr could not be detected at the exit port. This required about 20 min. The TFA solution was poured into 250 ml of anhydrous ether and the resulting slurry was stirred for 15 min. The HBr salt was collected, washed with Et2O, and dried in vacuo. The salt was dissolved in 15 ml of DMF and neutralized to pH 7.5 with N-methylmorpholine. The active ester, Z-Cys(Bz1)-ONp (0.67 g, 1.35 mmol), was added and the solution was stirred for 24 hr. The pH was readjusted to 7-7.5 with N-methylmorpholine and stirring was continued for 10 hr. At the end of this time a ninhydrin test of the reaction mixture was negative. The product was precipitated with 30 ml of H2O and 30 ml of EtOAc. The precipitate was collected, washed with H2O-EtOAc, EtOAc, Et2O and dried in vacuo: wt 1.12 g (89%); mp 235-236 °. The yellowish product was
dissolved in boiling HOAc. The product separated upon cooling and H₂O was added to complete the precipitation. The white precipitate was collected, washed with HOAc and Et₂O, and dried in vacuo: wt 0.77 g (61%); mp 238-239°; [α]²⁴° -37.3° (c 0.5, DMSO). From the filtrate an additional 0.15 g (mp 235-236°) was isolated for a total yield of 74%. Elemental analysis calculated for C₅₂H₆₄N₈O₁₂S₄: C, 59.06; H, 6.11; N, 10.60. Found: C, 59.26; H, 6.03; N, 10.32. The product showed a trace of a slower moving component on tlc on Silica Gel-G in the system 1-BuOH-HOAc-pyridine-H₂O (15:3:10:12).

**Synthesis of Z-Pro-Ile.** A solution of Z-Pro (5.0 g, 20 mmol) was prepared in 30 ml of THF containing 3 ml (21.5 mmol) of Et₃N and was cooled to -10°. Isobutyl chloroformate (2.8 g, 20.6 mmol) was added in 12 ml of THF and the milky emulsion was stirred for 20 min at -10°. Isoleucine (3.1 g, 24 mmol) dissolved in 26 ml of H₂O with 5 ml of Et₃N was added and the mixture was stirred 90 min at room temperature. The pH was adjusted to 2-3 by slow addition of conc. HCl to the cooled solution. The THF was removed by rotary evaporation and the resulting precipitate was filtered off, washed with 1 N HCl, and dried in vacuo: wt 7.2 g. The product was dissolved in 8 ml of HOAc and precipitated with 80 ml of H₂O: wt 6.5 g (89%); mp 126-129°. This was further recrystallized from CHCl₃-CCl₄ (10:90): wt 4.8 g (66%); mp 127-130°; [α]²⁴° -54.6° (c 0.4, MeOH). A second crop of 1.1 g (mp 122-126°) was isolated by concentrating the filtrate and precipitating the product with Et₂O and hexane for a total yield of 81%. Elemental
analysis calculated for $C_{19}H_{26}N_2O_5$: C, 62.95; H, 7.24; N, 7.73.
Found: C, 62.94; H, 7.32; N, 7.82.

Synthesis of Z-Pro-Ile-Gly-NH$_2$. A solution of Z-Pro-Ile (2.09 g, 5.76 mmol) in 15 ml of THF containing Et$_3$N (0.84 ml, 6 mmol) was cooled to -10°, and isobutyl chloroformate (0.79 g, 5.8 mmol) was added. After 20 min at -10°, a solution of glycinamide hydrochloride (0.65 g, 5.9 mmol) in 5 ml of H$_2$O containing 0.9 ml of Et$_3$N was added and the mixture was stirred for 90 min at room temperature. The solution was made acidic with a few drops of conc. HCl and the THF was removed by rotary evaporation. The aqueous solution was saturated with NaCl and the oily solid which separated was extracted into 1-BuOH. The BuOH phase was partially dried with Na$_2$SO$_4$. The remaining H$_2$O was removed as an azeotrope by rotary evaporation. Additional NaCl precipitated during the evaporation and was filtered off. The rest of the BuOH was removed by rotary evaporation and the oily residue was triturated to a solid with boiling EtOAc. After the suspension was cooled, the product was collected, washed with cold EtOAc, and dried in vacuo: wt 2.41 g; mp 150-154°, lit 178° (Jaquenoud and Boissonnas 1961). The crude product was recrystallized from H$_2$O-HOAc (15:1). Two crops of highly crystalline material were obtained totaling 1.27 g (53%); mp 179.5-181°. The product gave a single uniform spot on tlc on Silica Gel-G in the system 1-BuOH-HOAc-pyridine-H$_2$O (15:3:10:12).

Synthesis of Pro-Ile-Gly-NH$_2$. A solution of Z-Pro-Ile-Gly-NH$_2$ (1.05 g, 2.50 mmol) in 10 ml of 2.7 N HBr-HOAc was stirred for 55 min at room temperature. The HBr salt was precipitated by the addition of
anhydrous Et₂O, collected, washed with Et₂O, and dried in vacuo. The salt was dissolved in 1 ml of MeOH and passed through a 1 x 20 cm column of Rexyn 201 (OH⁻ cycle). The MeOH was removed from the eluate by rotary evaporation and the oil remaining was triturated to a solid in EtOAc-Et₂O (1:1). The product was collected, washed with Et₂O, and dried in vacuo: wt 0.61 g (86%); mp 165.5-169°. There is some confusion in the literature as to the correct melting point of this peptide. Jaquenoud and Boissonnas (1961) reported 118° and Rudinger et al. (1969) reported 168-171°. The product was further recrystallized for EtOH: wt 0.56 g (79%); mp 166-168°; [α]24D -56.2° (c 0.5, MeOH), lit. [α]D -63 ± 1° (HOAc), [α]D -67.8° (HOAc). Amino acid analysis (Spackman et al. 1958) after 24-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Pro, 1.02; Gly, 0.98; Ile, 1.01. Elemental analysis calculated for C₁₃H₂₄N₄O₅·½H₂O: C, 54.04; H, 8.56; N, 19.39. Found: C, 54.20; H, 8.25; N, 19.18. The compound gave a single spot of uniform shape on tlc on Silica Gel-G in the following systems: 1-BuOH-HOAc-pyridine-H₂O (15:3:10:12), 1-BuOH-HOAc-H₂O (7:1:2), and AmylOH-pyridine-H₂O (7:7:6).

Synthesis of Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Ile-Gly-NH₂. A solution of Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl) (1.12 g, 1.05 mmol), Pro-Ile-Gly-NH₂ (417 mg, 1.47 mmol), and 1-hydroxybenzotriazole (284 mg, 2.1 mmol) was prepared in 20 ml of DMF and cooled to 0°. Dicyclohexylcarbodiimide (260 mg, 1.26 mmol) was added. After stirring for 1 hr at 0°, the solution was allowed to warm to room temperature and the reaction was continued for 48 hr. The product was precipitated
with EtOH, collected, and washed with EtOH. The product was triturated in boiling EtOH and after cooling at 4°C overnight the product was again collected, washed with EtOH, and dried in vacuo: wt 1.10 g (79%); mp 253-254°C. The compound gave a single spot but with some tailing on tlc on Silica Gel-G in the system 1-BuOH-HOAc-H₂O-pyridine (15:3:12:10).

**Synthesis of Mesotocin.** In 200 ml of anhydrous liquid NH₃, Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Ile-Gly-NH₂ (399 mg, 0.30 mmol) was dissolved. A freshly cut piece of Na was dipped into the solution until the blue color persisted. After 3 min the excess Na was reacted with HOAc and the NH₃ was removed by evaporation and lyophilization. The residue was dissolved in 600 ml of deaerated 0.1% aqueous HOAc. The pH of the solution was adjusted to 8.5 with 3 N NH₃, and 62 ml of 0.01 N K₃[Fe(CN)]₆ was added. After 20 min the pH was adjusted to 4 with HOAc, and 5 ml of Rexyn 203 (Cl⁻ cycle) was added. After 15 min the resin was filtered off and washed with 0.2 N HOAc. A 50-ml portion of redistilled 1-BuOH was added to the filtrate to prevent foaming as the volume was reduced to 150 ml by rotary evaporation. The remaining aqueous solution was lyophilized to dryness. The crude product was dissolved in 5 ml of the upper phase and 3 ml of the lower phase of the system 1-BuOH-H₂O (1:1) (the aqueous phase 1.5% pyridine and 3.5% HOAc) and subjected to partition chromatography (Yamashiro 1964, Yamashiro et al. 1966) on a 2.9 x 65 cm column of Sephadex G-25 (100-200 mesh). The peptide material was detected by reading the absorbance of the eluate at 280 nm. The portion comprising the major peak at Rf 0.17 was isolated by lyophilization: wt 85.2 mg. This product was dissolved in 4 ml of
0.2 N HOAc and further purified by gel filtration (Porath and Flodin 1959) on a 2.9 x 64 cm column of Sephadex G-25 (200-270 mesh) equilibrated with 0.2 N HOAc. The product emerged as a single peak at 78% of the column volume and was isolated by lyophilization: wt 65.0 mg (20%); $\left[\alpha\right]_{546}^{24} -22.9^\circ$ (c 0.5, 1 M HOAc), lit. $\left[\alpha\right]_D^{24} -31.8^\circ$ (c 0.10-0.15, 1 M HOAc recalculated for anhydrous peptide). The product gave a single uniform spot on tlc using Silica Gel-G in the solvent systems used for Pro-Ile-Gly-NH$_2$ (see pages 80-81). Amino acid analysis after 24-hr hydrolysis in 6 N HCl at 110° gave the following molar ratios: Asp, 1.04; Glu, 1.02; Pro, 0.93; Gly, 0.99; Cys, 2.08; Ile, 1.91; Tyr, 1.02. Elemental analysis calculated for C$_{43}$H$_{63}$N$_{12}$O$_{12}$S$_2$: C, 51.27; H, 6.62; N, 16.69. Found: C, 51.06; H, 6.36; N, 16.75.

**Isolation of Tocinoic Acid.** After the mesotocin peak was completely eluted from the column used for partition chromatography ($R_f$ 0.10), the eluting solvent was changed from the upper phase of the solvent system to 20% aqueous HOAc and collection of the eluate was continued. Monitoring the absorbance at 280 nm showed a second major product emerging from the column. This material was isolated by lyophilization. The product, which was contaminated by much NaOAc, was dissolved in 5 ml of 0.2 N HOAc and subjected to gel filtration on a 2.9 x 61 cm column of Sephadex G-25 (100-200 mesh) equilibrated with 0.2 N HOAc. The peptide material was detected by reading the absorbance of the eluate at 280 nm. The product emerged from the coarse column as a sharp spike at 94% of the column volume preceded by three smaller, poorly resolved impurities. The major product was isolated by lyophilization and was found to still contain a significant amount of NaOAc.
The product was subjected to a second gel filtration on a 2.9 x 64 cm column of Sephadex G-25 (200-270 mesh) in 0.2 N H\textsubscript{2}O\textsubscript{Ac}. The product emerged at 93% of the column volume as a sharp peak preceded by two very small, well resolved impurities: wt 14.9 mg. This product gave a single spot of uniform shape identical to authentic tocinoic acid on tlc.

**Evaluation of the Synthesis**

The scheme of the synthesis was designed so that tocinoic acid, Pro-Ile-Gly-N\textsubscript{H}\textsubscript{2}, and mesotocin could be prepared from the same precursors. The results went one step beyond the goal when tocinoic acid was prepared concurrently with mesotocin. The fact that a good elemental analysis for Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Ile-Gly-N\textsubscript{H}\textsubscript{2} could not be obtained is explained by its being contaminated with some unreacted Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl) from which the tocinoic acid came. This simultaneous synthesis offered no problems in purification as mesotocin and tocinoic acid behaved quite differently in the partitioning system used. The mesotocin was eluted with the upper phase at $R_f$ 0.17 while the tocinoic acid had to be washed off the column with 20% aqueous H\textsubscript{2}O\textsubscript{Ac}.

**Pharmacological Activities of Mesotocin**

The oxytocic assay of mesotocin was performed on the uterus from a Sprague-Dawley rat which had received 100 μg of stilbestrol/day for three days (Follett and Bentley 1964) following the method of Holton (1948) and using modified Van Dyke-Hastings solution (Munsick
1960). The four-point assay design was used (Schild 1942). Sandoz preservative-free synthetic oxytocin was used at the concentration of 1 mg/ml with the nominal potency of 430 I.U./ml as the standard.

The oxytocic activity was found to be 320 ± 25 I.U./ml in the absence of Mg++. This was not significantly different from that reported by Berde and Boissonnas (1968), pp. 806-807). Nor was it significantly different from that reported by Rudinger et al. (1969) if a potency of 500 I.U./ml was assumed for their oxytocin standard. In the presence of 0.5 mmol of Mg++ the potency was increased by 1.27 times relative to oxytocin. This was slightly higher than the values reported for potentiation of mesotocin by Bentley (1965) 1.1, or by Rudinger et al. (1969) 1.15, but it was the same as the value reported by Munsick (1966) 1.3.

This preparation of mesotocin showed a milk ejecting activity which was identical within experimental error to that of crystalline deamino-oxytocin used as the standard. They were both active down to a concentration of $10^{-10}$ g/ml. The procedure used to measure the milk ejecting activity was similar to the one used by van Dongen and Hays (1966).

Currently the MRIF-like activity of mesotocin and its side chain, Pro-Ile-Gly-NH₂, are under investigation.
**APPENDIX A**

**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>Abbreviation</th>
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<tr>
<td>Benzyloxy carbonyl</td>
<td><img src="image" alt="benzyloxy carbonyl" /></td>
<td>Z</td>
</tr>
<tr>
<td>Benzyl</td>
<td><img src="image" alt="benzyl" /></td>
<td>Bz1</td>
</tr>
<tr>
<td>p-Bromobenzoyl</td>
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<td>Bz(Br)</td>
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<tr>
<td>p-Bromobenzyloxy carbonyl</td>
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<td>Z(Br)</td>
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<tr>
<td>p-Bromobenzyl</td>
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<tr>
<td>3,4-Dimethylbenzyl</td>
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<td>3,4-Me₂Bz1</td>
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<tr>
<td>p-Methoxybenzyl</td>
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<td>MeOBz1</td>
</tr>
<tr>
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<td>Formula</td>
<td>Abbreviation</td>
</tr>
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<td>--------------------</td>
<td>------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>tert-Butyloxycarbonyl</td>
<td>(CH$_3$)$_3$C-O-C-</td>
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**Carboxyl Protecting and Activating Groups**

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<tr>
<td>Benzyl ester</td>
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<tr>
<td>N-Hydroxysuccinimide ester</td>
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<td>ONSu</td>
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<tr>
<td>Methyl ester</td>
<td>CH$_3$O-</td>
<td>OMe</td>
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<tr>
<td>p-Nitrobenzyl ester</td>
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<td>ONB</td>
</tr>
<tr>
<td>p-Nitrophenyl ester</td>
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**Other Chemicals**

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<th>Formula</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>Acetic acid</td>
<td>CH$_3$-C-OH</td>
<td>HOAc</td>
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<tr>
<td>1-Butanol</td>
<td>CH$_3$-CH$_2$-CH$_2$-CH$_2$-OH</td>
<td>1-BuOH</td>
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<tr>
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<td>DCHA</td>
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<td>Formula</td>
<td>Abbreviation</td>
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<tr>
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<td>DCC</td>
</tr>
<tr>
<td>Dicyclohexylurea</td>
<td><img src="image2.png" alt="Image" /></td>
<td>DCU</td>
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<tr>
<td>Diethyl ether</td>
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<td>Et₂O</td>
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<tr>
<td>Diisopropylethylamine</td>
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<td>DIEA</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>(CH₃)₂N-C-H</td>
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</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>CH₃S-CH₃</td>
<td>DMSO</td>
</tr>
<tr>
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<td>CH₃CH₂OH</td>
<td>EtOH</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>CH₃COCH₂CH₃</td>
<td>EtOAc</td>
</tr>
<tr>
<td>β-Mercaptopropionic acid</td>
<td>H-SCH₂CH₂C-OH</td>
<td>β-MPA</td>
</tr>
<tr>
<td>Methanol</td>
<td>CH₃OH</td>
<td>MeOH</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Na⁺O-C-CH₃</td>
<td>NaOAc</td>
</tr>
<tr>
<td>Name</td>
<td>Formula</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------</td>
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</tr>
<tr>
<td>Tetrahydrofuran</td>
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</tr>
<tr>
<td>Triethylamine</td>
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<td>$\text{Et}_3\text{N}$</td>
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<tr>
<td>Trifluoroacetic acid</td>
<td>$\text{CF}_3\text{-C-OH}$</td>
<td>TFA</td>
</tr>
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</table>
APPENDIX B

PUBLICATIONS OF AUTHOR RELATED TO THIS WORK
PLEASE NOTE:

Pages 91-93, "The Synthesis and Some Pharmacological Properties of Tocinoic Acid and Desinotocinoic Acid\(^{1,2}\)", copyright 1972 by the American Chemical Society, and pages 94-95, "Melanophore Stimulating Hormone: Release Inhibition by Ring Structures of Neurohypophysial Hormones", copyright 1972 by the American Association for the Advancement of Science, not microfilmed at request of author. Available for consultation at the University of Arizona Library.

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