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A Rapid, Efficient Synthesis of Oxytocin and 8-Arginine-vasopressin. Comparison of Benzyl, *p*-Methoxybenzyl, and *p*-Methylbenzyl as Protecting Groups for Cysteine

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Oxytocin and 8-arginine-vasopressin have been synthesized in high yields in a short time using 1.5-fold molar excesses of protected amino acids by means of solid-phase synthesis on a benzhydrylamine resin. Coupling of each residue to the peptide resin was measured by automated picrate monitoring. High-pressure liquid partition chromatography was found to be extremely useful in characterizing products and by-products. Benzyl, *p*-methoxybenzyl, and *p*-methylbenzyl were compared as cysteine protecting groups in the syntheses, with the last two being preferred.

Introduction and Strategy

The neurohypophyseal hormones and their synthetic analogues have been the subject of many studies aimed at elucidating their physiological properties and the correlation between their structures and functions,[1] with oxytocin (I, Figure 1) receiving particular attention in these investigations. We are presently concerned with developing an approach to the unequivocal determination of the conformations and dynamic properties of peptides in solution, principally concentrating our investigations on oxytocin. The technique which we are employing in these investigations is nuclear magnetic resonance (NMR), measuring three-bond homo- and heteronuclear coupling constants from which dihedral angles and their rotational isomerism can be deduced. To extract such data from the spectra, a variety of isotopic isomers is needed. Selective deuteration is required to simplify overlap and coupling in the proton NMR spectra, and selective enrichments in ^{13}C and ^{15}N facilitate the observation of couplings to these less abundant nuclei.[2] Because of the number of isomers needed for a complete study and the expense of enriched precursors, we have undertaken development of methods for the rapid and efficient synthesis of oxytocin. Our strategy for these studies is to synthesize a number of isomers of oxytocin with simultaneous isotopic enrichments in several of the residues. Our synthetic goal is a generally optimized procedure using all the amino acid reagents with maximum

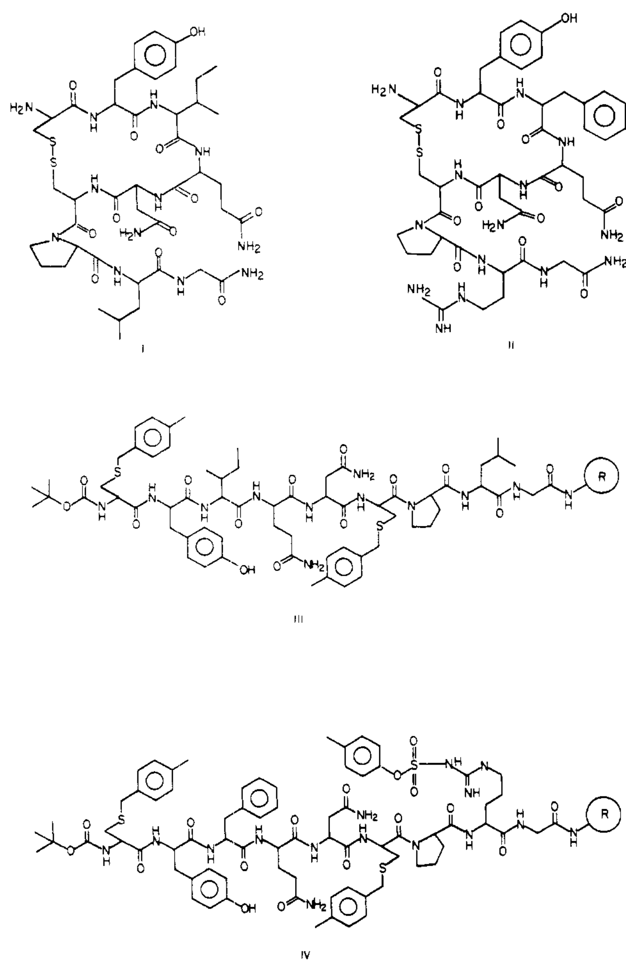

Figure 1. Structures of compounds described.

efficiency, while providing the greatest yield of final product.

Comparisons of previous synthetic routes to oxytocin and some of its analogues can be made on the basis of reported results. Solution peptide synthesis methods and solid-phase methods using chloromethylated resin have given comparable efficiencies on the basis of the quantities of individual amino acid reagents consumed.[3] A comparison of solid-phase syntheses of an oxytocin analogue with benzhydrylamine and with chloromethyl resins indicates that the former resin gave superior results. Treatment of the protected peptide–benzhydrylamine resin with anhydrous hydrogen fluoride yields the desired peptide amide directly,[4] saving time and avoiding losses that might be incurred in the conversion of the terminal carboxyl group to the desired carboxamide, as would be necessary subsequent to cleavage from chloromethyl resin. We have chosen the solid-phase method with benzhydrylamine resin, since it is less time consuming than the other alternatives and should provide a greater yield. A significant additional advantage of the solid-phase approach is the availability of a quantitative nondestructive procedure using picric acid, by which the extent of coupling can be easily monitored spectrophotometrically.[5] This has allowed us to optimize coupling conditions with reasonable effort.

Since several of the amino acids in oxytocin are trifunctional (Cys^1, Tyr^2, Gln^4, Asn^5, Cys^6), it is necessary to consider the effect of protecting groups on the yield of the synthesis. The advantages of side-chain protection in the overall synthesis of the peptide must be balanced against any losses that arise in the derivatization of an isotopically enriched amino acid. Asparagine and glutamine protected at the α-amino group with *tert*-butoxycarbonyl (Boc) and without side-chain protection can be coupled effectively during solid-phase synthesis of an analogous peptide in dimethylformamide (DMF) using dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole monohydrate (HOBzt).[3] Deprotection of protected tyrosine at the conclusion of a synthesis can lead to side reactions,[6] but tyrosine can be used without protecting the phenolic hydroxyl group, particularly in the synthesis of oxytocin, where it is the penultimate residue incorporated.[7] Since isotopic isomers of these three amino acids can be readily synthesized in an unprotected form,[8] we decided to employ them with only α-amino protection in the peptide synthesis.

Cysteine is the only amino acid for which side-chain protection is absolutely necessary in this synthesis. Benzyl (Bzl) and *p*-methoxybenzyl (*p*-MeOBzl) are the sulfhydryl protecting groups most commonly used for this purpose. The more stable benzyl group, though often used in amino acid and peptide synthesis, requires rather vigorous conditions for removal.[9] In our hands these conditions reduced the yield of oxytocin by a factor of 2 compared to that achieved using *p*-MeOBzl protection. However, the *p*-MeOBzl protecting group could not be used successfully in the routes we employed for synthesis of isotopic isomers of cysteine,[8] and conversion from one protecting group to the other after completion of the amino acid synthesis is only about 70% efficient. The *p*-methylbenzyl (*p*-MeBzl) group has been suggested for use in peptide synthesis because it may be removed in hydrogen fluoride under milder conditions.[6] In addition, it has been found to be a good protecting group to use in amino acid synthesis as well.[8] We chose to use the *p*-MeBzl group and found the results of the peptide synthesis to be comparable to those obtained with *p*-MeOBzl protection.

After some investigation we devised a peptide synthetic scheme in which 1.5-fold excesses of amino acid reagents are employed in single couplings for Gly^9, Leu^8, Pro^7, Cys^6, Tyr^2, and Cys^1, and two such couplings are employed for Asn^5, Gln^4, and Ile^3. The only side-chain protection used is the *p*-MeBzl group on both half-cystyl residues. Though the nature and origin of the synthetic by-products are unclear, an important feature of this synthesis is that they are easily separated from the desired product in a single gel filtration, which also serves the purpose of desalting the final products. The overall procedure from the start of the solid-phase synthesis to recovery of chemically pure, fully active product in 55% yield (relative to glycine substitution on the resin) can be carried out in four working days. The final product was characterized by bioassay, amino acid analysis, counter-current distribution, high-pressure liquid chromatography, high-resolution proton NMR, thin-layer chromatography, and optical rotation.

This synthesis of oxytocin has given reproducible results a number of times using labeled and unlabeled precursors. We wished to determine whether the considerations we had applied to increasing efficiency here were readily applicable to the synthesis of another peptide. Therefore, 8-arginine-vasopressin (AVP, II) was prepared in the same manner as oxytocin with minimal changes in procedure. We used tosyl protection for the guanidino group of arginine in addition to *p*-MeBzl on the half-cystyls. From the picrate monitoring data, it appears that satisfactory results are obtained with single 1.5-fold excess couplings for all but Asn^5, Gln^4, and Tyr^2. The final purification of AVP from this synthesis is as easy as that of oxytocin.

Experimental Section

Materials. N^{α}-*tert*-butoxycarbonyl-L-amino acids were obtained from Beckman, Bachem, and Chemical Dynamics Corp., except for Boc-*S*-*p*-methylbenzylcysteine, which was made at The Rockefeller University by Dr. Wesley Cosand. All were checked for purity by

thin-layer chromatography (TLC) (see Analytical Methods). All amino acids (except glycine) were of the L configuration. Methylene chloride (technical, Eastman) was distilled from Na_2CO_3 (anhydrous reagent, Baker). Dimethylformamide (DMF) (spectroquality, MCB) was stored over molecular sieves (4A, Chemical Dynamics) and tested for amines before use in synthesis.[10] Diisopropylethylamine (DIEA) (Aldrich) was distilled after reflux overnight with CaH_2. 1-Butanol (reagent, Baker), acetone (reagent, Baker), and trifluoracetic acid (TFA) (Halocarbon) were distilled before use. Picric acid (0.1 M) solutions were made up by dissolving picric acid (reagent, MCB) in distilled CH_2Cl_2, stirring with anhydrous Na_2SO_4, and filtering before use.

The following reagents were of reagent grade, if more than one grade was available, and were used as received: acetic acid (Baker); isopropyl alcohol (Baker); ethyl acetate (Baker); dicylohexylcarbodiimide (DCC) (Pierce); 1-hydroxybenzotriazole monohydrate (Aldrich); Sephadex G-15 dextran gel (Pharmacia); ethanol (IMC Chemical Group); fluorescamine (Hoffmann-La Roche); and anhydrous HF (Matheson).

Analytical Methods. Final characterization of oxytocin and AVP was carried out using bioassay, amino acid analysis, counter-current distribution, high-resolution proton NMR, high-pressure liquid chromatography in a system known to resolve oxytocin from 14 replacement and deletion analogues, TLC in three systems, and optical rotation. TLC of peptides was carried out on analytical silica gel G plates (Analtech) with the following solvent systems: (A) 1-butanol–acetic acid–water (4:1:5, upper phase); (B) 1-butanol–acetic acid–pyridine–water (15:3:10:12); (C) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Material on the plates was visualized by ninhydrin or chlorine–*o*-toluidine reaction. TLC of amino acids was performed on similar plates with chloroform–methanol–acetic acid (85:10:5). Counter-current distribution (CCD), both analytical and preparative, was carried out on a 100-tube CCD apparatus (Post Scientific) with 10 mL of each phase per tube. The solvent system used was 1-butanol–ethanol–0.5% acetic acid in water (4:1:5).[11]

High-pressure liquid chromatography (HPLC) was based on the system of Gruber et al.[12] The equipment we used consisted of a glass linear-gradient maker, a high-pressure pump (Milton Roy), a high-pressure injection valve (Waters), a Partisil-10 ODS prepacked column 0.4 × 25 cm (Whatman), a Spectro/Glo fluorometer (Gilson), and a strip chart recorder (Easterline Angus). A 15–95% acetone–water gradient was run for 75 min at a rate of 0.8 mL/min. Samples were prepared by dissolving them in 0.4 mL of 0.046 M sodium phosphate, pH 7, in a disposable borosilicate test tube, and then adding 0.2 mL of fluorescamine solution (20 mg/100 mL of acetone) with Vortex mixing. A pH 7 standard buffer (Beckman) was found to be a convenient source of the 0.046 M sodium phosphate. After 10 min the sample was diluted up to volume with a solution of 0.03% ammonium formate and 0.01% thiodiglycol. Amino acid analyses were carried out after the procedure of Spackman, Stein, and Moore[13] on a Beckman model MS amino acid analyzer using a 0.9 × 30 cm column of Durrum DC-6A resin. The analyzer had been modified to perform automatically the two buffer changes that are required for a single-column analysis. The Durrum pico-buffer system II was employed. Hydrolysis was carried out in culture tubes, with teflon-lined caps, that were inserted into a heating block. Peptide samples were hydrolyzed in 12 M HCl–acetic acid–liquified phenol[14] (2:1:1) or in 6 M HCl. The latter conditions were used after performic acid treatment of the peptide for conversion of cysteine to cysteic acid.[15] Hydrolyses were carried out for 24 h at 110 °C. When the first conditions were used, the hydrolysate was extracted three times with $CHCl_3$ before drying and then diluted in 0.2 M pH 2.2 citrate buffer for application to the column. In the second case the hydrolysate was dried directly and diluted similarly.

Peptide resin hydrolysis was carried out by two procedures. Either the 12 M HCl–acetic acid–liquified phenol (2:1:1) method described above was used, with the addition that the hydrolysate was filtered before extraction, and the resin was washed with several small portions of 1 M HCl, or 12 M HCl–propionic acid (1:1) at 135 °C for 12 h was used.[16] These latter samples were filtered, and the resin was washed with 1 M HCl as above before drying and subsequent dilution.

Proton NMR spectra were obtained on a Varian HR/NTC TT-220 spectrometer. Optical rotations were measured on a Cary 60 spectropolarimeter, using sucrose as a standard.

***tert*-Boc-*S*-*p*-MeBzl-Cys-Tyr-Ile-Gln-Asn-*S*-*p*-MeBzl-Cys-Pro-Leu-Gly-NH_2 Resin (III).** A 1.0-g sample of benzhydrylamine hydrochloride resin from polystyrene–1% divinylbenzene (Beckman lot no. B1135) was placed in a 75-mL reaction vessel of a Schwartz/Mann peptide synthesizer. Analysis of the resin by Beckman indicated 0.53 mequiv of N/g of resin by elemental analysis and 0.48 mequiv/g available amine via Boc-L-proline coupling, HF cleavage, hydrolysis, and amino acid analysis. We also monitored coupling capacity using HCl-propionic acid hydrolysis of fully coupled glycinamide resin with the result of 0.48 mequiv of available coupling sites/g of resin and using picrate monitoring of the deprotected glycinamide resin, giving 0.50 mequiv/g of resin. Amino acids were dissolved in their appropriate solvents (the Boc derivatives of *p*-MeBzl-cysteine, glycine, proline, leucine, and isoleucine in CH_2Cl_2, asparagine and glutamine in DMF with a twofold molar excess of HOBzt, and tyrosine in 5% DMF in CH_2Cl_2) and placed in the appropriate reservoirs in the synthesizer. The synthesis was initiated at step 7 in Table I and carried through as indicated in Tables I and II until the final coupling of Boc-*p*-MeBzlCys (1.5-fold molar excess, single couplings for Gly, Leu, Pro, Cys, and Tyr, and double couplings for Asn, Gln, and Ile). The monitoring procedure (Table III) was incorporated as desired after step 15 of Table I or step 19 of Table II. The final seven washes of the monitoring sequence were collected automatically, and the absorbance at 362 nm was determined on a Zeiss PMQ II spectrophotometer. The ϵ_{362} for the DIEA–picrate is

Table I. Coupling Scheme for Residues Gly[9], Leu[8], Pro[7], Cys[6], Ile[3], Tyr[2], and Cys[1] [e]

Step	Reagent	Volume, mL	Duration, min	No. of times
1	CH_2Cl_2	25	0.5	5
2	50% TFA–CH_2Cl_2	25	2.0	1
3	50% TFA–CH_2Cl_2	25	30.0[a]	1
4	CH_2Cl_2	25	0.5	5
5	2-Propanol	17	0.5	2
6	CH_2Cl_2	25	0.5	5
7[b]	5% DIEA–CH_2Cl_2	17	2.0	3
8	CH_2Cl_2	25	0.5	5
9[c]	Amino acid–CH_2Cl_2	5	2.0	1
10	DCC–CH_2Cl_2	5	30.0	1
11	CH_2Cl_2	25	0.5	5
12	2-Propanol	17	0.5	2
13	CH_2Cl_2	25	0.5	2
14	2-Propanol	17	0.5	2
15[d]	CH_2Cl_2	25	0.5	5

[a] Deprotection time for Gln[4], before Ile[3] coupling, was 15 min. [b] For the coupling of Gly[9] to the resin the sequence is started at this step. [c] The vessel is not drained after this step. [d] Steps 9–15 were repeated when a second coupling was performed for Ile[3]. [e] Amino acids were *tert*-butoxycarbonyl derivatives in 1.5 M excess in solution except tyrosine, which was dissolved in 5% DMF-CH_2Cl_2. DCC was equimolar with protected amino acids.

Table II. Coupling Scheme for Residues Asn[5] and Gln[4] in Dimethylformamide[c]

Step	Reagent	Volume, mL	Duration, min	No. of times
1–8 are identical to those in Table I				
9	2-Propanol	17	0.5	2
10	CH_2Cl_2	25	0.5	5
11[a]	DMF	17	2.0	3
12[b]	Amino acid HOBzt–DMF	5	2.0	1
13	DCC–DMF	5	120.0	1
14	DMF	17	2.0	1
15	CH_2Cl_2	25	0.5	5
16	2-Propanol	17	0.5	2
17	CH_2Cl_2	25	0.5	2
18	2-Propanol	17	0.5	2
19	CH_2Cl_2	25	0.5	5

[a] When second couplings were used, steps 11–19 were repeated. [b] The vessel is not drained after this step. [c] Amino acids were *tert*-butoxycarbonyl derivatives in 1.5-fold molar excess in DMF solution. HOBzt was used at a twofold molar excess over amino acid concentrations; DCC was equimolar.

Table III. Monitoring Scheme[a]

Step	Reagent	Volume, mL	Duration, min	No. of times
1	DIEA 5% in CH_2Cl_2	17	2.0	3
2	CH_2Cl_2	25	0.5	5
3	2-Propanol	17	0.5	2
4	CH_2Cl_2	25	0.5	5
5	0.1 M picric acid in CH_2Cl_2	17	2.0	3
16	CH_2Cl_2	25	0.5	2
7	2-Propanol	17	0.5	2
8	CH_2Cl_2	25	0.5	5
9	5% DIEA in CH_2Cl_2	17	2.0	3
10	CH_2Cl_2	25	0.5	4

[a] Washes in steps 9 and 10 were collected and their absorbance was measured at 362 nm.

15 000 (Table III).[5] At the conclusion of these steps 1.530 g of air-dried III was recovered (96% yield). Amino acid analysis of III after hydrolysis in the HCl–acetic acid–phenol mixture was Asp 1.00, Glu 1.03, Pro 1.05, Gly 1.10, Ile 0.93, Leu 1.03, and Tyr 0.92. The amino acid analysis showed no loss of chains from the resin during synthesis. Syntheses using between 1 and 2 g of resin have been carried out using the same protocols and with similar proportional yields.

***tert*-Boc-*S*-*p*-MeOBzlCys-Tyr-Ile-Gln-Asn-*S*-*p*-MeOBzlCys-Pro-Leu-Gly-NH_2 Resin (V)** and ***tert*-Boc-*S*-BzlCys-Tyr-Ile-Gln-Asn-*S*-BzlCys-Pro-Leu-Gly-NH_2 Resin (VI)** were prepared similarly to III, except that the appropriately S-derivatized Boc-cysteine was used. Variations in number of couplings or molar excesses of amino acids used made no detectable difference in yield of peptide resins (by weight) or in their amino acid analyses.

Oxytocin (I). A. 0.439-g sample of III was placed in a 50-mL Teflon-Kel-F vessel used on an HF apparatus (Toho) described elsewhere.[10] A small Teflon-coated magnetic stirring bar and 1 mL of anisole were added. A frit was secured near the top of the vessel and the vessel was attached to the apparatus. The apparatus was evacuated with an aspirator pump, and the sample vessel was immersed in a dry ice/acetone bath. After 20 min the sample vessel was disconnected from the vacuum and connected to the HF reservoir, and HF was distilled into the vessel until the total liquid volume was approximately 10 mL. (This process takes about 15 min.) The dry-ice bath was then replaced by a water–ice bath (0 °C) with a magnetic stirrer underneath, and the sample vessel was sealed off from the rest of the system. After 75 min the vessel was carefully opened to the aspirator and the HF was allowed to evaporate. Virtually all the HF was gone after 10 or 15 min. After 30 min of aspiration, the line was switched to a mechanical pump and pumping continued for 1 h. The sample remained immersed in a 0 °C bath throughout this time.[17] The system was then filled with Ar or N_2 to atmospheric pressure, and the cleavage vessel was quickly removed and sealed with parafilm.

The material was washed out of the vessel into a coarse fritted funnel with several portions of degassed ethyl acetate totaling about 100 mL. The funnel and the vessel were then placed in a large lyophilizer vessel and evacuated for 30 min to remove remaining ethyl acetate. The cleavage vessel and the resin in the funnel were then washed with several portions of degassed 1 M acetic acid (80 mL total), followed by 160 mL of degassed distilled water in several portions. The solution was then adjusted to pH 8 with 3 M NH_4OH, and 25 mL of 0.01 M potassium ferricyanide solution was added in order to form the disulfide bond of oxytocin. The yellow solution was stirred for about 30 min. The solution was then adjusted to pH 5 with 50% acetic acid, and AG-3 anion-exchange resin (TFA^- form) was added and the mixture stirred for an additional 20 min. The slurry was then filtered, yielding a clear colorless solution, and the resin was washed with a small portion of water. The solution was then lyophilized.

After lyophilization the powder was taken up in about 10 mL of 50% acetic acid and filtered through a millipore filter, yielding a clear, pale yellow solution that was applied to a Sephadex G-15 (2.5 × 70 cm) column previously equilibrated with 50% acetic acid.[18] The column was run at a flow rate of 1 mL/min and 7-mL fractions were collected. Two peaks were eluted as determined by monitoring at 280 nm. Peak 1 retained the yellow color and appeared in fractions 15–18, and peak 2, which was subsequently determined to be pure I, was in fractions 19–26. There was some overlap of the two peaks. Fractions that showed significant amounts of both materials present as judged by TLC were subjected to further purification with CCD. (This was not necessary in the case being described.) Peak 2 yielded 76 mg of material (55% yield) from peptide resin, and peak 1 yielded 30 mg (22% yield). TLC of the peak 1 material in the manner described above in solvent system A gave a poorly resolved series of bands with R_f's between 0.17 and 0.35. The ninhydrin color was purple. Peak 2 gave a yellow spot (by ninhydrin reaction) when TLC was performed using solvent systems A, B, and C. The TLC results agree with those obtained for a sample of oxytocin supplied to us. R_f in system A was 0.34; chlorine-*o*-toluidine visualization did not reveal any additional spots. Amino acid analyses for these materials are: Asp 1.00, Glu 0.90, Pro 1.07, Gly 1.07, Cys 1.30, Ile 0.77, Leu 1.05, and Tyr 0.78 for peak 1; and Asp 1.00, Glu 1.02, Pro 1.08, Gly 0.97, Cys 1.89, Ile 0.93, Leu 0.99, and Tyr 1.05 for peak 2. The peak 2 material gave only one peak in the appropriate fractions when subjected to CCD.[11] On HPLC the fluorescamine-derivatized peak 2 material appeared as a single peak at a position in agreement with that of a sample of oxytocin supplied to us. The material in peak 1 was retained more strongly on the Partisil ODS column and showed several components, apparently including some oxytocin. This was indicated by the TLC as well. Proton NMR spectra were determined in D_2O and were in complete agreement with previously reported results.[19] Bioassay of peak 2 material for avian vasopressor activity gave a result of 416 ± 19 U/mg, in good agreement with the literature value of 450 ± 30 U/mg,[20] $[\alpha]^{27}{}_{589}$ − 22° (*c* 0.48, in acetic acid) [lit.[3c] $[\alpha]^{22.5}{}_{D}$ − 24° (*c* 0.5, in acetic acid)].

Cleavage of up to 2 g of resin has been carried out using separate cleavage vessels on the HF apparatus and then combining the material for subsequent workup with no significant effect on overall yield.

I was derived from V in the same way as from III and the results were virtually identical.

I was derived from VI by the same procedure, except that the cleavage vessel was immersed in a bath at 20 °C rather than 0 °C. We detemined by NMR studies of cleaved VI that these conditions were required for complete deprotection of the Bzl groups in this system. Yield of oxytocin (I) was 25% for this workup from VI.

***tert*-Boc-*S*-*p*-MeBzlCys-Tyr-Phe-Gln-Asn-*S*-*p*-MeBzlCys-Pro-N^g-TosArg-Gly-NH_2 Resin (IV)** was prepared in the same manner as III with the following differences. Boc-N^g-tosylarginine, dissolved in 5% DMF in CH_2Cl_2, was the second amino acid coupled, and Boc-phenylalanine dissolved in CH_2Cl_2 was the seventh amino acid coupled. A single coupling with 1.5-fold excess of glycine was followed by three 30-min couplings each with 1.5-fold excesses for arginine and proline, two couplings at 1.5-fold excess for *p*-MeBzl-cysteine (the first for 30 min and the second for 60 min), and two 120-min couplings at 1.2-fold excess (due to an instrument adjustment) for asparagine and glutamine. The last three residues were coupled in the manner of the first cysteine. IV (1.06 g) was obtained (92% yield). Picrate monitoring results are in Table V. Amino acid analysis of IV was Asp 1.00, Glu 0.97, Pro 0.89, Gly 1.09, Tyr 0.85, Phe 0.83, and Arg 0.88.

8-Arginine-vasopressin (II) (AVP) was made from IV following the same procedure used for I; 0.72 g of IV yielded 130 mg (52% yield) of II in peak 2 following gel filtration. Peak 1 contained 65 mg (26% yield). TLC analysis of the material in peak 1 showed a broad band between R_f's 0.0 and 0.2 (purple ninhydrin reaction) and a single yellow spot R_f 0.2 for peak 2 material in solvent system A. Peak 2 material gave single yellow spots in systems B and C, and the results in all three systems were consistent with those obtained from a sample of AVP supplied to us. NMR spectra were in agreement with those previously reported.[21] HPLC is illustrated in Figure 2 with the single peak for derivatized G-15 peak 2 material. The result on our material was consistent with that for AVP supplied to us. Rat pressor activity was 496 ± 44 U/mg, in good agreement with the literature value of 487 ± 15 U/mg.[7] Amino acid analysis result for peak 1 material was Asp 1.00, Glu 0.91, Pro 0.98, Gly 1.00, Cys 1.44, Tyr 0.85, Phe 0.84, and Arg 1.07, and for peak 2 Asp 1.00, Glu 0.93, Pro 0.97, Gly 0.99, Cys 1.96, Tyr 0.9, Phe 0.94, and Arg 0.95, $[\alpha]^{27}{}_{589}$ −23° (*c* 0.23, in acetic acid) [lit.[7] $[\alpha]^{22}{}_{D}$ −22° (*c* 0.22, in acetic acid)].

Results and Discussion

From the results in the Experimental Section it is clear that benzhydrylamine-derivatized polystyrene resin provides an excellent support for solid-phase synthesis of these neurohypophyseal peptide hormones. It offers a more stable peptide–resin linkage which permits the use of fairly vigorous conditions for deprotection of α-amino groups (<0.4% loss of glycine/h on treatment with 50% TFA in CH_2Cl_2), while affording efficient peptide–resin cleavage (>95%) in anhydrous

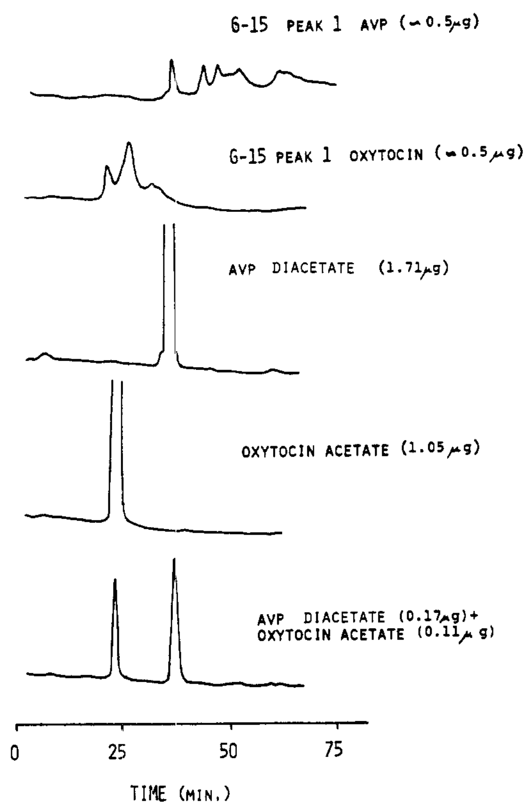

Figure 2. High-pressure liquid chromatography of fluorescamine-derivatized products of oxytocin and 8-arginine-vasopressin syntheses. The by-products of the oxytocin synthesis gave similar chromatograms regardless of protecting group used. The ordinate is intensity of fluorescence, and the traces are displaced, but are on the same scale. The bottom three traces are of G-15 peak 2 material.

hydrogen fluoride at 0 °C for 60 min. Couplings appear to proceed to completion using standard synthetic methods with considerably less than the usual molar excesses of amino acids. This is illustrated by the results of amino acid analyses given in the previous section and by the data in Tables IV and V, where the results of picrate monitoring of the oxytocin and vasopressin syntheses are presented. The data in these tables may be usefully assessed in terms of effective coupling efficiency, that is, the ratio of the percentage completion for a given set of conditions to that after efforts to force completion. Confirmation of the reliability of the effective coupling efficiency can be observed in the similarity of the peptide resin amino acid analyses and synthetic results for syntheses where both 1.5-fold and threefold excesses of amino acid reagents have been used. The appearance in these tables of values significantly less than 100% after both initial 1.5-fold excess couplings as well as after attempts to force the reaction to completion may indicate picrate binding to unreacted free amino termini or to unidentified sites. This background level of picrate eluted after efforts to complete coupling was found to increase with chain length. One possible source of the background would be the appearance of chains with unreactive, but picrate positive, amino group. The increasing level of background during the synthesis is consistent with these chains remaining unreactive throughout the synthesis. Alternatively, some other binding of picrate to the growing peptide chain may be taking place. The trends in the monitoring results for both the oxytocin and vasopressin syntheses were similar, although the background levels for AVP were somewhat higher. We ultimately recovered comparable amounts of material from each synthesis, and this speaks against the possibility that the increased background was due to a larger number of chain terminations. If deletions were

Table IV. Coupling Efficiencies (%) in the Synthesis of Oxytocin

Residue	With 1.5-fold excess single coupling	With threefold excess double coupling	Effective coupling efficiency for single 1.5-fold excess coupling[a]
Gly^9	99.5	99.6	99.9
Leu^8	99.3	99.5	99.8
Pro^7	99.6	99.4	100.2
Cys^6	99.4	99.3	100.1
Asn^5	93.5	99.3	94.2
Gln^4	97.5	98.2	99.3
Ile^3	90.0	98.3	91.6
Tyr^2	97.6	98.0	99.6
Cys^1	97.0	96.8	100.2

[a] Ratios of value in column 2 to those in column 3 (see text), expressed as percentage.

Table V. Coupling Efficiencies (%) in the Synthesis of AVP

Residue	With 1.5-fold excess single coupling	With 1.5-fold excess double coupling	Effective coupling efficiency for single 1.5-fold excess coupling[a]
Gly^9	99.6	99.6	100.0
Arg^8	95.6	96.2 (96.6)[b]	99.4
Pro^7	92.5	94.7 (95.0)[b]	97.4
Cys^6	91.3	92.7	98.5
Asn^5	68.0	93.6	72.6
Gln^4	58.7	90.3	65.0
Phe^3	88.7	89.3	99.3
Tyr^2	80.2	89.5	89.6
Cys^1	93.0	93.7	99.3

[a] Ratios of values in column 2 to those in column 3 (see text), expressed as percentage. [b] A result after third coupling (see text).

present in our purified material, they should probably have appeared as additional peaks in the high-pressure liquid chromatograms.[22] However, no significant additional peaks were present for either oxytocin or AVP. The by-products in peak 1 from the Sephadex G-15 column from both syntheses are presumably higher molecular weight materials; if they contain deletion peptides, the ultimate fate of these species must have been the formation of dimers or oligomers through intermolecular disulfide bonds. The reproducibility of the monitoring data indicates that the factors giving rise to the background are intrinsic to the synthesis.

We have also employed the minitoring method to determine reasonable coupling times for Boc-glutamine and Boc-asparagine in DMF with HOBzt and DCC. Under the conditions we are using, the reactions are about 50% complete in 30 min, and virtually totally complete in 120 min. The same batch of resin was used for all the syntheses of oxytocin, and the reproducibility of the minitoring data demonstrates the applicability of this technique for quality control in such repeated syntheses.

In considering the different yields obtained from syntheses using the three different protecting groups for cysteine, we note that there is no evidence for any variation of the products in synthesis of the protected oxytocin peptide resin as a function of the cysteine protecting group used. The Bzl group with its substantial stability has been used effectively in amino acid and peptide synthesis, and it can be removed completely by exposure to hydrogen fluoride at 20 °C for 60 min in this system. The *p*-MeOBzl group is the most labile[6,9] and provides a point of comparison with the other two, even though

for reasons given earlier it is not ideal for our needs. The yield of oxytocin with Bzl protection under these conditions is about half as great as that obtainable with *p*-MeOBzl protection. The ratios of material in peak 1 (by-product) to that in peak 2 (oxytocin) from the gel filtration are quite similar for both means of protection, as are the TLC, HPLC, and amino acid analysis results for the respective peaks. The *p*-MeBzl group, though not widely used in peptide synthesis, has lived up to the expectations for it mentioned earlier, and the synthetic results with this group are indistinguishable from those with *p*-MeOBzl protection. Further efforts were made to determine whether any more of the desired product could be regenerated from peak 1 of the gel filtration or whether a change in our procedures could increase the yield of oxytocin further. An obvious property of the peak 1 material is a substantial reduction in cysteine content relative to that in oxytocin. The level of cysteine, however, showed no significant variation with protecting group and cleavage conditions, or with sequence. Several other possible causes of this effect were investigated. To check for sulfoxide formation as a source of the problem, acetone treatment in hydrogen bromide–acetic acid was used to reduce any *p*-MeBzl-cysteine sulfoxide to *p*-MeBzl-cysteine.[22] Some of the peak 1 material was treated with acetone after being dissolved in 35% hydrogen bromide–acetic acid. This was followed by treatment with hydrogen fluoride at 0 °C for 60 min, oxidation with performic acid, hydrolysis, and amino acid anaylsis. The results were the same as before. Alternatively, it is possible that deprotection before the coupling of Cys[6] or Cys[1] was incomplete. Employing double deprotections before these couplings, however, did not affect the final results. To test a third alternative we removed the terminal Boc group before hydrogen fluoride cleavage, but also to no avail.

A possible source of some of the peak 1 material is suggested by the amino acid analysis results for the peptide resin and for peak 1, namely that some termination at glutamine occurs during the synthesis. Comparison of tyrosine, isoleucine, and, in the case of AVP, phenylalanine values with that of cysteine indicate that this can only be part of the problem.

In total we are able to account finally for between 85 and 90% of the peptide from the protected peptide resin, with 55– being the desired product, 25% peak 1 by-products, 5% remaining associated with the resin, and possibly 5% lost by termination at glutamine. We have attempted to optimize the synthesis of oxytocin and have succeeded in developing a procedure that is efficient in both time and material. The readily purified product is obtained in a yield which is substantially greater than previously reported (~30%[3]), and the approach appears to be applicable to analogous peptides. Effectively complete couplings can be achieved without resorting to a large excess of reagents or long coupling times. These conditions are particularly important to us because of our interest in preparing labeled peptides.The possibility that these conditions may work well in general is of practical interest to anyone contemplating large scale syntheses. The picrate monitoring method has proved indispensable in assessing the effectiveness of couplings in this work.

The syntheses reported here demonstrate convincingly the value of using *p*-methylbenzyl protection of the cysteine thiol function. The previously suggested[6] value of this protecting group based on amino acid model studies is thus supported by its actual use in peptide synthesis. Since the protected amino acid is easily prepared from readily available materials and has clear advantages over *S*-benzyl- and *S*-*p*-methoxybenzylcysteine, we consider that it is the protecting group of choice in this case.

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Registry No.—*p*-MeBzl-cysteine, 42294-52-0; glycine, 56-40-6; proline, 147-85-3; leucine, 61-90-5; isoleucine, 73-32-5; asparagine, 70-47-3; glutamine, 56-85-9; tyrosine, 60-18-4; oxytocin, 50-56-6; Boc-Cys($CH_2C_6H_4OMe$-H)OH, 18942-46-6; Boc-Cys(CH_2Ph)-OH, 5068-28-0; *tert*-Boc-*S*-*p*-MeBzlCys-Tyr-Ile-Gln-Asn-*S*-*p*-MeBzlCys-Pro-Leu-Gly-NH_2, 63534-39-4; Boc-*N*-tosylarginine, 13836-37-8; Boc-phenylalanine, 13734-34-4; 8-Arginine-vasopressin, 113-79-1.

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