Bidirectional Solid-Phase Peptide Synthesis. Extension of the Dinitrophenylene-Bridging Method to Cysteine-Containing Peptides

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Abstract: Procedures have been established for the attachment of N-protected cysteine derivatives to solid supports through an *S*-dinitrophenylene bridge. Solid-phase peptide synthetic techniques have been modified to allow elaboration of peptide chains in both the amino- and carboxyl-terminal directions from the resin-anchored cysteine residue. Release of the completed cysteine-containing peptides from the solid support is achieved by thiolysis of the *S*-dinitrophenylene bond. Oxytocin and deamino-oxytocin have been prepared as a demonstration of the applicability of this method of bidirectional solid-phase peptide synthesis to cysteine-containing peptides.

Recent studies in this laboratory demonstrated that the dinitrophenylene (DNPene) group can act as a covalent bridge between solid supports and the imidazole function of histidine residues. Histidine derivatives, thus anchored through their side chains to solid matrices, served as nuclei for the bidirectional solid-phase synthesis of histidine-containing peptides[1,2] (Figure 1, AA-His). Removal of the peptide from the resin was achieved by thiolysis. Advantages deriving from the versatility of the bidirectional synthesis and from the mild conditions of the peptide-resin scission recommend the DNPene attachment as an alternative to the standard[3-5] system of solid-phase peptide synthesis.

The susceptibility of *S*- and *O*-dinitrophenyl (DNP) derivatives of cysteine and tyrosine, respectively, to cleavage by thiols[6] suggested that the general technique of bidirectional DNPene bridging might be used for the synthesis of peptides containing cyst(e)ine and tyrosine in addition to those containing histidine (Figure 1). The synthesis of cyst(e)ine-containing peptides is of particular interest in this laboratory (*e.g.*, ref 7–9); therefore, the development of *S*-DNPene-bridging techniques and of methods for bidirectional peptide synthesis from the resulting resin-anchored cysteine derivatives was undertaken.

Attachment of *tert*-butyloxycarbonylcysteine to supporting resin through an *S*-DNPene linkage was analogous to the corresponding step for attachment of *tert*-butyloxycarbonylhistidine.[1,2] Thus *tert*-butyloxycarbonylcysteine, in the presence of Et_3N, reacted with a fluorodinitrophenylglycinylpolystyrene–2% divinylbenzene (FDNP-Gly-*O*-resin) to yield a Boc-Cys-(DNP-Gly-*O*-resin)-OH compound. In preliminary studies, resin-anchored *S*-dinitrophenylcysteine derivatives, in the presence of excess Et_3N, showed some tendencies toward the β elimination[10] (Figure 2) and S → N migration[11] (Figure 3) reactions. Since both of these side reactions occur more readily under more basic conditions, treatment of the peptide-resin compounds with excess Et_3N (as in the standard Merrifield cycle for conversion of peptide hydrochloride and trifluoroacetate salts to the free bases) was abandoned in favor of neutralization methods involving less basic reagents. Treatment of peptide-resin salts with an appropriate mixture of *N*-methylmorpholine and acetic acid in DMF ("buffered DMF") was found to render the peptide-resin derivatives susceptible to acylation without apparent base-catalyzed side reactions at the *S*-DNP bridgehead, as judged on the basis of yield and quality of products. The use of acetate buffer for neutralization of peptide-resin salts necessitated, in turn, modifications in subsequent acylation steps. Thus, the presence of acetic acid or acetate salts precluded treatment of the peptide-resin compounds with DCCI or other carboxyl-activating reagents. All acylations involving peptide-resin derivatives neutralized with buffered DMF were accomplished with activated esters or other "preactivated" acylating agents.

The foregoing modifications led to the development of a bidirectional, solid-phase method of synthesizing cyst(e)ine-containing peptides as demonstrated by the synthesis of the neurohypophyseal hormone, oxytocin,[12] and its highly potent analog, deamino-oxytocin.[13,14] The synthetic routes from the resin-bound *tert*-butyloxycarbonylcysteine derivative to oxytocin and deamino-oxytocin are depicted in Figure 4. Boc-Cys-(DNP-Gly-*O*-resin)-OH was coupled with H-Pro-Leu-Gly-NH_2 through the action of DCCI–HOSu in DMF solution.[15] Through successive acidolytic deprotec-

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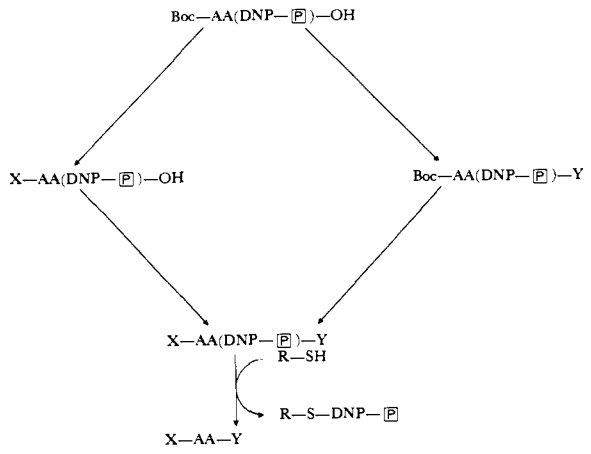

Figure 1. General scheme of bidirectional solid-phase peptide synthesis on dinitrophenylene resins. –AA– = amino acid residue with a side chain which forms a thiol-labile dinitrophenylene derivative; P = solid support; X = N-terminal limb of peptide or peptide derivative; Y = C-terminal limb of peptide or peptide derivative.

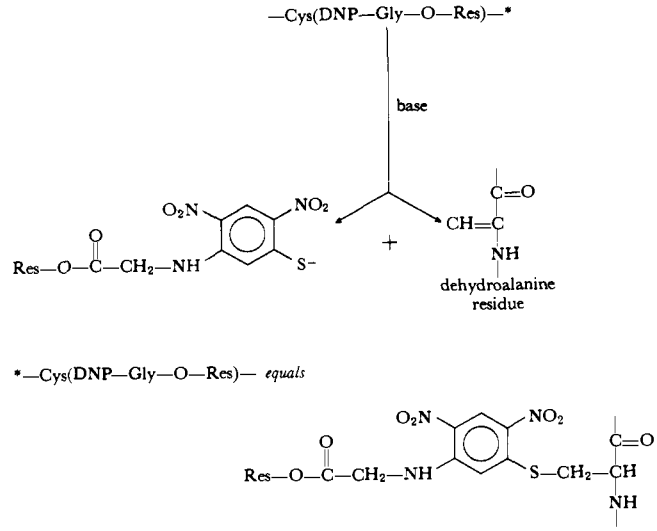

Figure 2. β-Elimination reactions of *S*-DNP-resin-anchored cysteine derivatives.

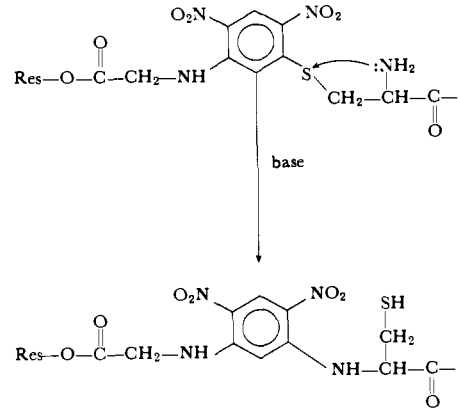

Figure 3. S → N migrations of *S*-DNP-resin-anchored cysteine derivatives.

tions, treatments with buffered DMF, and acylations with appropriate *tert*-butyloxycarbonylamino acid derivatives, the resultant Boc-Cys(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2 was converted stepwise to Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2.[16] The latter intermediate was N-deprotected, neutralized with buffered DMF, and coupled with Z-Cys(Bzl)-ONp to yield the corresponding resin-bound nonapeptide of oxytocin. For the synthesis of deamino-oxytocin the resin-bound octapeptide was elongated by addition of β-Mpr(Bzl)-OH in the presence of DCCI–HOBt. Treatment of the peptide-resin compounds with 2-mercaptoethanol in buffered DMF yielded partially protected deamino-oxytocin and oxytocin derivatives, respectively. These were converted to the corresponding hormone and hormone analog by treatment with sodium in boiling anhydrous ammonia,[17] followed by standard methods for oxidative formation of the requisite disulfide bonds. The hormonal peptides were purified by partition chromatography[18] on Sephadex G-25. Deamino-oxytocin was subsequently crystallized.[14] Both oxytocin and deamino-oxytocin were characterized by amino acid composition, chromatographic behavior, optical rotation, and by bioassay.

The foregoing syntheses of oxytocin and deamino-oxytocin demonstrate the applicability of the dinitrophenylene-bridging method to the bidirectional synthesis of cysteine-containing peptides. It should be noted that the partially protected peptide intermediates obtained by thiolytic release from the polymeric support are not strictly defined chemical entities but are presumably isolated as a mixture of free sulfhydryl compounds, dimers, and mixed disulfides with 2-mercaptoethanol. This view is consistent with our observation that hydrolysates of the intermediates yield low cystine values; yet, hydrolysates of performic acid-oxidized samples exhibit the expected cysteic acid content. Although the partially protected cyst(e)ine-containing intermediates are not subject to all analytical criteria of purity, they are easily and efficiently converted in good yield and purity to the chemically well-defined, biologically active peptides.

The use of the *N*-methylmorpholine–acetic acid mixture in DMF in this study instead of Et_3N represents a departure from the standard Merrifield method of conversion of the peptide-resin salts to the free bases. Both procedures serve to convert the strongly protonated amino group of the resin-bound peptides to a readily acylatable form. On the one hand, Et_3N neutralization attempts to convert the peptide resin as completely as possible to the free base by vigorous scavenging of protons. On the other hand, *N*-methylmorpholine, a weaker base than Et_3N to begin with, is partially protonated in the presence of acetic acid in DMF solution. Quite possibly such "buffered DMF solutions" do not convert the peptide-resin salts completely to the free base but instead to a mixture of free base and its acetate. In any event, the buffered DMF solution effects the conversion of the trifluoroacetate or hydrochloride to an acylatable form in a considerably less basic environment than excess Et_3N in DMF.

Buffered DMF solutions may also be useful for other aspects of peptide synthesis. For example, the proton protection of guanidino groups of Arg-containing

(16) From the amino acid composition of Boc-Asn-Cys(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2 hydrolysates, there is no indication of S → N migration competing with the acylation of H-Cys(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2.

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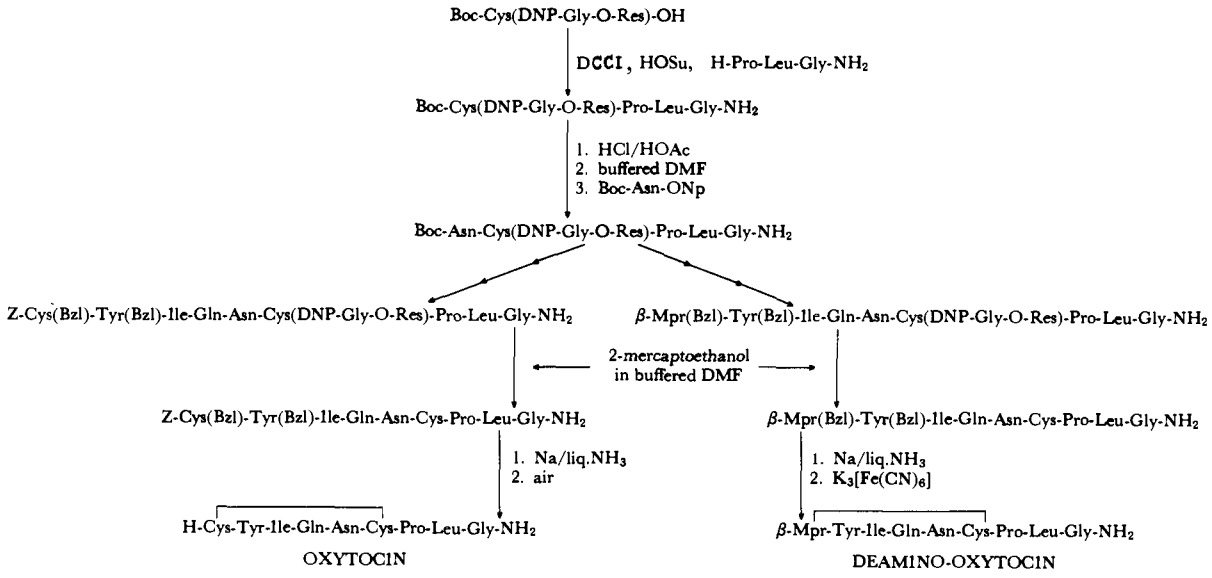

Figure 4. Syntheses of oxytocin and deamino-oxytocin starting with Boc-Cys(DNP-Gly-*O*-res in)-OH.

peptides, common in classical peptide chemistry (for discussion, see ref 19), might be practical in solid-phase peptide synthesis with reliable differential protonation of amino and guanidino functions. Also, the difficulties with the use of the DNP group as protection for the histidine side chain in solid-phase peptide synthesis[20,21] may be avoidable by substitution of buffered DMF solutions for excess Et_3N in the neutralization of peptide-resin salts.

Some of the more obvious advantages of the *S*-DNPene-bridging method for bidirectional solid-phase synthesis of peptides have been put forward in discussions of the dinitrophenylene bridging method in general.[1,2] Projects currently underway seek to capitalize on the bidirectional potential inherent to DNPene-bridging methods and on the mildness of the peptide-resin cleavage, along with the speed and convenience inherent in solid-phase synthetic methods generally. Such bidirectional methods lend themselves particularly to the synthesis of cyclic peptides, to the introduction of preformed carboxyl derivatives (*e.g.*, esters or substituted amides) into the C termini, to the introduction of acid-labile moieties into the C termini of peptides, and, generally speaking, to the synthesis of peptide-analog series with structural variations in the C-terminal regions. In this context, Z-Cys(Bzl)-Tyr-(Bzl)-Ile-Gln-Asn-Cys(DNP-Gly-*O*-resin)-Pro-OH has already been prepared and extended on the C terminal with several dipeptide amides to give a series of 8- and 9-substituted oxytocin analogs for biological, enzymological, and conformational investigations. In addition to the advantages deriving from the bidirectionality of the synthetic methods and from the mildness of the scission of the peptide-resin linkage, we envision potential for novel uses of the *S*-dinitrophenylene bridging methods based on the peculiar chemistry of *S*-dinitrophenylcysteine derivatives. The β-elimination reaction in strong base of *S*-DNPene-resin derivatives is being examined as a synthetic route to dehydroalanine-containing peptides; these compounds, in turn, can be converted to peptide amides by hydrolytic or oxidative cleavage of the dehydroalanine residues.[22,23] In our continued effort we will attempt to combine the mild conditions of the dinitrophenylene attachment methods with the use of protecting groups removable under comparably mild experimental conditions with the aim of achieving a uniformly mild overall peptide synthetic method.

Experimental Section

"Buffered DMF." The relative strength of several bases in DMF was determined by the procedure of Pearson and Vogelsang[24] using Bromcresol Green rather than 2,4-dinitrophenol as a spectrophotometric indicator. According to this method the base strengths were of the order Et_3N > phenylalanine amide, tyrosine ethyl ester, and *N*-methylmorpholine > tribenzylamine. These results are in line with those of Williams and Young[25] for Et_3N, *N*-methylmorpholine, and tribenzylamine studied in a number of organic solvents other than DMF. Bromcresol Green was also used to indicate the degree of availability of protons with various *N*-methylmorpholine–acetic acid mixtures in DMF. For this purpose a series of DMF solutions was prepared with fixed concentrations of *N*-methylmorpholine (1 *M*) and indicator but with varying amounts of HOAc. Absorbance of the solutions was measured at 614 nm and the results as given in Figure 5 reveal that a DMF solution 1 *F* in *N*-methylmorpholine and 0.5 *F* in HOAc (referred to as "buffered DMF") can serve as a buffer. The ability of this solution to act as a buffer in the presence of hydrochloride salts was tested by monitoring possible changes in absorbance of constant amounts of Bromcresol Green in "buffered DMF" contining 0–160 μmol/ml of glycine ethyl ester hydrochloride. No measurable changes in absorbance were observed over the entire concentration range of hydrochloride.

Boc-Cys(DNP-Gly-*O*-resin)-OH. *tert*-Butyloxycarbonylcysteine (20 mmol)[26] was dissolved in a mixture of 15 ml of MeOH, 20 ml of HOAc, and 5 ml of H_2O. Zinc powder (5 g) was added to the

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(20) J. T. Potts, Jr., G. W. Tregear, H. T. Keutmann, H. D. Niall, R. Sauer, L. J. Deftos, B. F. Dawson, M. L. Hogan, and G. D. Aurbach, *Proc. Nat. Acad. Sci. U. S.*, **68**, 63 (1971).

(21) During the synthesis of TRH by the DNPene method we observed that resin derivatives featuring an N^{im}-DNPene function undergo a severe, irreversible darkening on contact with excess Et_3N, accompanied by reduced yield of product. Other researchers have indicated in informal discussions similar observations with N^{im}-DNP-histidine-containing peptides.

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solution in several portions over a 2-hr period. The solution was filtered and evaporated to yield a gummy residue. The crude product was taken up in 50 ml of deaerated H_2O, the pH of the solution was adjusted to *ca.* 2 with HCl, and the aqueous solution was extracted with three 5-ml portions of deaerated benzene. Thin-layer chromatography of the extracted material on silica gel G in the system $CHCl_3$–MeOH–HOAc (40:2:1, v/v/v) revealed only one major spot staining with Cl_2/KI–starch, trace amounts of *tert*-butyloxycarbonylcysteine, and no ninhydrin-positive components. The benzene solution was evaporated to a gummy residue which was taken up in deaerated DMF. This solution, which contained approximately 15 mmol of *tert*-butyloxycarbonylcysteine determined according to the method of Ellman,[27] was added to a F-DNP-Gly-*O*-resin compound, which was prepared as described previously[1,2] from a Boc-Gly-*O*-resin (10 g) substituted at 0.28 mmol of Gly/g of resin. Et_3N (2.8 mmol) was added in three portions over a 6-hr period to the resin suspension. After 24 hr the resin compound was washed with DMF, EtOH, and Et_2O and then dried *in vacuo*; yield, 12 g.

An analytical sample of the Boc-Cys(DNP-Gly-*O*-resin)-OH was suspended for 24 hr in buffered DMF containing 10% (v/v) of 2-mercaptoethanol. The resin was recovered by filtration and resubjected to thiolysis for another 24 hr. The combined thiolysates were taken to dryness, oxidized with performic acid, treated with 1 *N* HCl in HOAc, and reevaporated. Measurement of cysteic acid by amino acid analysis indicated a substitution of 0.20 mequiv of Cys/g of resin compound.

β-Mpr(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 Fraction. Boc-Cys(DNP-Gly-*o*-resin)-OH (3.0 g) was suspended in 15 ml of DMF and crystalline H-Pro-Leu-Gly-$NH_2 \cdot 0.5H_2O$ (2.0 mmol),[28] HOSu (5.1 mequiv), and DCCI (2.0 mequiv) were added. The canary yellow resin changed to a red-brown color over a period of about 3 hr and then to bright yellow on shaking overnight. Assembly of the N-terminal limb of the resin-bound, protected deamino-oxytocin derivative was accomplished by stepwise addition of acyl or *tert*-butyloxycarbonylaminoacyl units following a cycle of deprotection, neutralization, and coupling operations separated by washing procedures. Thus, prior to the removal of the amino-protecting group each Boc-peptide-resin intermediate was washed successively with three 15-ml portions of DMF, EtOH, and HOAc. The washed resin derivative was then treated with 15 ml of 1 *N* HCl in HOAc (or with 15 ml of TFA in the case of the intermediate terminating in Boc-Gln) for 30 min. For deprotection of the Ile residue, the treatment with 1 *N* HCl in HOAc was repeated. The resulting peptide-resin hydrochloride (or trifluoroacetate) salt was washed successively with three 15-ml portions of HOAc, EtOH, and DMF. Neutralization of peptide salts was achieved by brief shaking with the buffered DMF solution and the resulting peptide-resin intermediates were washed quickly with two 15-ml portions of DMF. The washed derivatives were immediately treated with appropriately activated compounds for acylation of the liberated amino groups.

The asparagine, glutamine, and tyrosine residues were introduced into the growing peptide chain by the *p*-nitrophenyl ester method[29,30] using Boc-Asn-ONp,[31,32] Boc-Gln-ONp,[33] or Boc-Tyr(Bzl)-ONp[34] (5.1 mequiv each). For incorporation of Boc-Ile[35] and β-Mpr(Bzl)[36] into the appropriate peptide intermediate, DCCI[37] was used as coupling reagent in the presence of 1-hydroxybenzotriazole (HOBt).[38] To this end, the carboxylic acid (5.1 mmol) was dissolved with 10.2 mmol of HOBt in 7.5 ml of DMF at 0°. DCCI (3.8 mmol) was added to the solution with stirring. After about 15 min the reaction mixture was allowed to warm to room temperature and to stir for another hour. The resulting solution, with suspended DCU, was transferred with another 7.5 ml of DMF to the vessel containing the deprotected, neutralized, and washed peptide resin derivative. Coupling reactions by either the nitrophenyl ester or the DCCI–HOBt method were allowed to proceed with shaking for about 20 hr.

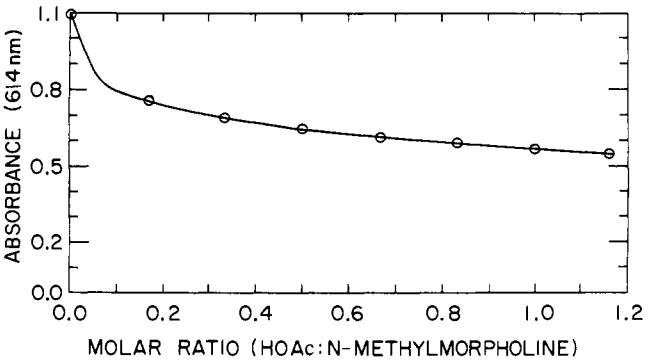

Figure 5. Buffering effect of *N*-methylmorpholine–acetic acid mixtures in dimethylformamide solution. Changes of absorbance of Bromcresol Green (monitored at 614 nm) in dimethylformamide containing 1 *M* *N*-methylmorpholine in the presence of increasing concentrations of acetic acid.

After the N-terminal limb of the peptide chain had been extended stepwise according to the foregoing procedures to yield β-Mpr-(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2, the peptide-resin compound was washed successively with three 15-ml portions of DMF, EtOH, and Et_2O and then air-dried. The resin was suspended in 30 ml of DMF buffer containing 10% by volume of 2-mercaptoethanol. After 24 hr the resin was removed from the suspension by filtration and resubjected to thiolytic treatment for 24 hr. The resin was again collected by filtration and washed successively with three 15-ml portions of DMF, HOAc, and DMSO. The filtrates from the two thiolysis treatments were combined with the washings, and the pooled filtrates and washings were added slowly to 600 ml of water with stirring. The precipitated product was collected by centrifugation, the centrifugal pellet was frozen, and the product was obtained as a white powder (584 mg) by lyophilization of the frozen pellet. The powder was dissolved in 3 ml of DMSO and the filtered solution was added slowly to a stirred bath of absolute ethanol (100 ml). The precipitate which separated from this solution was collected by centrifugation and washed first with EtOH and H_2O, and then recovered as a white powder (406 mg, 58% of the theoretical yield based on the original substitution of the resin by Boc-Cys-OH) by lyophilization from a frozen centrifugal pellet.

Samples of the purified peptide intermediate were hydrolyzed *in vacuo* for 22 hr at 110° in 6 *N* HCl. Amino acid analysis of the hydrolysates according to the method of Spackman, *et al.*,[39] gave ninhydrin-reactive products in the molar ratios: Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; Ile, 0.9; Leu, 1.0; Tyr, 0.9; 0.5Cys, 0.7; NH_3, 3.1. Analysis of a sample which had been oxidized by performic acid[40] gave a value of 1.1 for $CysO_3H$.

Various analytical procedures were carried out to monitor the progress of the synthesis. Ninhydrin assays[41] on small aliquots of peptide-resin intermediates before and after each coupling reaction served to test for completion of each acylation step. The H-Cys-(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2 compound failed to give a ninhydrin reaction by the Kaiser method; therefore, its acylation with Boc-Asn-ONp could not be monitored in this manner. A small amount of the Boc-Asn-Cys(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2 intermediate was hydrolyzed for 22 hr at 110° in a 12 *N* HCl–dioxane mixture (1:1, v/v) and the hydrolysate was dried and subjected to amino acid analysis. The following molar ratios were Asp, 0.9; Pro, 0.8; Leu, 1.0; Gly, 1.0.

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 Fraction. The synthesis of Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(DNP-Gly-*O*-resin)-Pro-Leu-Gly-NH_2 followed that of the intermediate of

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(40) C. H. Li, *J. Biol. Chem.*, **229**, 157 (1957).
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deamino-oxytocin, except that Z-Cys(Bzl)-ONp[42] was substituted for the activated β-Mpr(Bzl) derivative in the final coupling step. The peptide-resin compound was washed successively with DMF, EtOH, and Et_2O and then air-dried. A portion of the peptide-resin product (15%) was reserved, and the remainder of the material was suspended for 24 hr in 30 ml of buffered DMF containing 10% 2-mercaptoethanol. The resin was collected by filtration and re-subjected to thiolysis for an additional 24 hr. Again the resin was removed from the suspension by filtration and it was washed with two 15-ml portions of DMF. The combined thiolysis filtrates and DMF washings were added slowly to a stirred 300-ml bath of H_2O. The precipitated product was collected by centrifugation, washed with water, and recovered as a white powder (479 mg) by lyophilization of a frozen centrifugal pellet. Part of the lyophilized powder (379 mg) was dissolved in a few milliliters of HOAc, freed of insoluble material by filtration, and added slowly to a stirred bath (200 ml) of ethanol. The precipitated product was collected by centrifugation, washed successively with ethanol and water, and then recovered as a white powder (290 mg, 54%) by lyophilization of a frozen centrifugal pellet. Samples of the purified peptide intermediate were hydrolyzed for 22 hr *in vacuo* at 110° in 6 *N* HCl, with and without prior performic acid oxidation. Amino acid analysis of the hydrolysates showed ninhydrin-positive materials in the molar ratios: Asp, 1.0; Glu, 1.0; Gly, 0.9; Pro, 1.2; Ile, 1.0; Leu, 1.0; Tyr, 0.9; 0.5Cys (unoxidized sample), 0.6; or $CysO_3H$ (performic-oxidized sample), 1.0; and NH_3, 3.1.

Deamino-Oxytocin. β-Mpr(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (200 mg) was treated with sodium metal in boiling anhydrous ammonia until a blue color persisted for 10–15 sec. Excess sodium was quenched with HOAc, and the ammonia was allowed to evaporate. The residual reduced peptide was dissolved in 200 ml of water containing a few drops of acetic acid, the pH of the solution was adjusted to about 8 with ammonia, and the solution was treated with potassium ferricyanide.[36] After about 30 min the solution was treated with an excess of Dowex AG 3 X4 in the TFA form for removal of iron salts. The ion-exchange resin was removed by filtration and crude hormone analog was recovered from the filtered solution by lyophilization. The material was subjected to partition chromatography on a 2.0 × 50 cm bed of Sephadex G-25 in the solvent system *n*-BuOH–benzene–pyridine–HOAc–H_2O (50:50:1.5:3.5:95). The major elution peak, as determined by Folin–Lowry assay,[43] was centered at R_f 0.20. Deamino-oxytocin has been reported to exhibit in this chromatographic system an R_f of 0.19.[14] Of the 63 mg of purified deamino-oxytocin recovered from partition chromatography, 25 mg was dissolved in 0.8 ml of hot water and then left to crystallize according to Ferrier, *et al.*[14] Crystalline deamino-oxytocin (20.7 mg, corresponding to 16% based on the initial substitution of the resin by Boc-Cys-OH) was recovered by filtration, washed with a few drops of cold water, and dried *in vacuo* at room temperature: mp 179–183°; $[\alpha]^{23}D$ −94.4° (*c* 0.5, 1 *N* acetic acid) [lit.[44] $[\alpha]^{25}D$ −95.1° (*c* 0.5, 1 *N* acetic acid)]. When the analog was subjected to avian vasodepressor assay using the four-point design on four conscious chickens[45] according to the procedure of Coon[46] an activity of 970 units/mg was recorded (the value is based on anhydrous peptide). The physical, chemical, and biological properties of the crystalline deamino-oxytocin are in good agreement with previously reported data.[14]

A hydrolyzed sample of deamino-oxytocin gave the following molar ratios of ninhydrin-active components: Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; $CysO_3H$ (determined with a performic acid-oxidized sample), 1.0; Ile, 0.9; Leu, 1.0; Tyr, 0.9; and NH_3, 3.0.

Oxytocin. Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (200 mg) was reduced with metallic sodium in boiling anhydrous ammonia as described above. The reduced peptide, after evaporation of ammonia, was dissolved in 200 ml of water containing a few drops of acetic acid. The pH of the solution was adjusted to about 6.8 with ammonia, and the solution was aerated for about 6 hr, at which time it was negative to the Ellman test for sulfhydryl groups. Crude hormone was recovered by lyophilization and purified by partition chromatography on Sephadex G-25 in the system *n*-BuOH–pyridine–HOAc–H_2O (1000:15:35:950). Oxytocin (102 mg) was recovered from fractions corresponding to the major Folin–Lowry peak which was centered at an R_f of 0.21. A 34.8-mg aliquot of oxytocin was subjected to gel filtration on a Sephadex G-25 column using 0.2 *N* HOAc as eluting solvent and by this procedure 25.5 mg (27% theoretical yield) of oxytocin was recovered: $[\alpha]^{23}D$ −23.9° (*c* 0.5, 1 *N* acetic acid) [lit. $[\alpha]^{22.5}D$ −23.1° (*c* 0.5),[47] $[\alpha]^{24}D$ −24.0° (*c* 0.51),[26] $[\alpha]^{20}D$ −24.3° (*c* 0.45);[48] all in 1 *N* acetic acid]. The purified oxytocin possessed an avian vasodepressor activity of 480 units/mg. A hydrolyzed aliquot of oxytocin gave upon amino acid analysis the following molar ratios of ninhydrin-active compounds: Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; 0.5Cys, 2.2; Ile, 1.0; Leu, 1.0; Tyr, 1.0; and NH_3, 2.9.

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