Human Pituitary Growth Hormone. 36. The Solid-Phase Synthesis of the Carboxyl Terminal Cyclic Dodecapeptide

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The COOH-terminal cyclic dodecapeptide of human growth hormone Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe (I) has been synthesized by the solid-phase method. New amino acid derivatives, N^{α}-Boc-*S*-3,4-dimethylbenzylcysteine and N^{α}-Boc-γ-*p*-bromobenzylglutamic acid, were synthesized and used in a second synthesis of I. The corresponding linear carbamidomethyl derivative, Val-Gln-Cys-(CH_2CONH_2)-Arg-Ser-Val-Glu-Gly-Ser-Cys(CH_2CONH_2)-Gly-Phe (II) has also been synthesized. Peptide I was readily converted to peptide II by reduction of I with dithiothreitol and alkylation with iodoacetamide.

One of the two disulfide bridges in the human growth hormone (HGH) molecule is present in a small loop near COOH-terminal phenylalanine.[1,2] During the course of total synthesis[3] of HGH-like protein, we synthesized by the solid-phase procedure[4] the COOH-terminal cyclic dodecapeptide Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe (I).[5] Subsequent examination of protecting groups[6] for cysteine and glutamic acid suitable for solid-phase synthesis of proteins has now led to the use of the 3,4-dimethylbenzyl group for cysteine and the *p*-bromobenzyl for glutamic acid in the synthesis of I as reported herein. Previous studies showed that the disulfide bridges are unnecessary for the biological activity of the hormone since the reduced tetra-S-carbamidomethylated HGH is as active as the natural hormone.[7] It is, therefore, of interest to synthesize the corresponding linear carbamidomethyl derivative Val-Gln-Cys(CH_2CONH_2)-Arg-Ser-Val-Glu-Gly-Ser-Cys(CH_2CONH_2)-Gly-Phe (II). The synthesis of II is also reported herein.

For the synthesis of I (Chart I) N^{α}-Boc protection was employed along with the following side-chain protecting groups: cysteine, *p*-methoxybenzyl; serine, benzyl; glutamic acid, *p*-bromobenzyl; and arginine, tosyl. Since γ-benzyl protection of glutamic acid was reported[8] to not be entirely stable under conditions for deblocking the Boc group, we explored the use of *p*-bromobenzyl protection which is about four times as stable as benzyl protection.[9] The choice of tosyl protection for arginine has recently been discussed[10] Since the present work was initiated before introduction of the modified Loffet procedure[11,12] for attachment, it should be noted that the starting Boc-Phe resin used in the syntheses reported herein was prepared by the triethylamine procedure.[4] Removal of the protecting groups from the dodecapeptide and cleavage of the peptide from the solid support were performed with hydrogen fluoride.[13] Oxidation of the disulfhydryl dodecapeptide[14] was accomplished by the use of ferricyanide as previously employed[15] for deaminooxytocin. Purification by gel filtration on Sephadex G-25 was followed by chromatography on carboxymethylcellulose[16] (Figure 1). Cyclic dodecapeptide I was isolated in highly purified form as indicated by thin layer and paper chromatography, paper electrophoresis, and amino acid analysis. Evidence that the synthetic product was a monomer was obtained by partial reaction of the single amino group with 2,4-dinitrofluorobenzene[17] and examination of the products by electrophoresis.

It has been reported that the *p*-methoxybenzyl group can more readily be removed from *p*-methoxybenzylcysteine by hydrogen fluoride than the benzyl group.[8,18] However, for synthesis of very large peptides by the solid-phase procedure, a high degree of stability to repeated deblocking conditions is demanded for side-chain protection in addition to its facile removal at the end of the synthesis. Thus, N^{α}-acetyl-*S*-(*p*-methoxybenzyl)cysteinamide[6] was prepared and its stability tested by treatment with 50% trifluoroacetic acid in dichloromethane for 23 hr at 24°. Determination of sulfhydryl groups liberated showed that 27% of the protection was lost. This degree of instability suggests that this protecting group, if used in conjunction with N^{α}-Boc protection, might not be suitable for very large

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(2) C. H. Li and J. S. Dixon, *Arch. Biochem. Biophys.*, **146**, 233 (1971); C. H. Li, *Proc. Amer. Phil. Soc.*, **116**, 365 (1972).

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(5) Symbols and abbreviations are in accordance with the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature, *Biochemistry*, **11**, 1726 (1972). Other abbreviations used are HGH, human growth hormone; CMC, carboxymethylcellulose; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide.

(6) A preliminary account of some of the work in this paper has been reported previously: D. Yamashiro, R. L. Noble, and C. H. Li, in "Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 197.

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(8) B. Gutte and R. B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971).

(9) When the influence of the amino and α-carboxyl groups is removed, the benzyl protection is more stable (see ref 6).

(10) D. Yamashiro, J. Blake, and C. H. Li, *J. Amer. Chem. Soc.*, **94**, 2855 (1972).

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(14) Attempts to form the disulfide bridge by air oxidation failed although reduced HGH is oxidized by air with ease [T. A. Bewley and C. H. Li, *Arch. Biochem. Biophys.*, **138**, 338 (1970)]. A similar difficulty in ring closure for deaminooxytocin led to the development of ferricyanide as oxidant (see ref 15).

(15) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, **237**, 1563 (1962).

(16) E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.*, **78**, 751 (1956).

(17) A. R. Battersby and L. C. Craig, *J. Amer. Chem. Soc.*, **73**, 1887 (1951).

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CHART I
OUTLINE OF THE SYNTHESIS OF I.

Boc-Phe-OCH_2 resin

↓ 1. TFA–CH_2Cl_2 2. diisopropylethylamine–$CHCl_3$ 3. Boc-Gly, DCC

Boc-Gly-Phe-OCH_2 resin

↓ etc.

Boc-Val-Gln-Cys(*p*-MeOBzl)-Arg(Tos)-Ser(Bzl)-Val-Glu(*p*-BrBzl)-Gly-Ser(Bzl)-Cys(*p*-MeOBzl)-Gly-Phe-OCH_2 resin

↓ 1. HF, 0°, 1 hr

H-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe-OH

↓ 2. $K_3Fe(CN)_6$, pH 8 3. gel filtration on Sephadex G-25 4. CMC

H-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe-OH

I

CHART II
OUTLINE OF THE SYNTHESIS OF II

Boc-Phe-OCH_2 resin

↓ 1. TFA–CH_2Cl_2 2. diisopropylethylamine–$CHCl_3$ 3. Boc-Gly, DCC

Boc-Gly-Phe-OCH_2 resin

↓ etc.

Boc-Val-Gln-Cys(CH_2CONH_2)-Arg(Tos)-Ser(Bzl)-Val-Glu(*p*-BrBzl)-Gly-Ser(Bzl)-Cys(CH_2CONH_2)-Gly-Phe-OCH_2 resin

↓ 1. HF, 0°, 1 hr 2. gel filtration on Sephadex G-25 3. partition chromatography on Sephadex G-25 4. CMC

H-Val-Gln-Cys(CH_2CONH_2)-Arg-Ser-Val-Glu-Gly-Ser-Cys(CH_2CONH_2)-Gly-Phe-OH

II

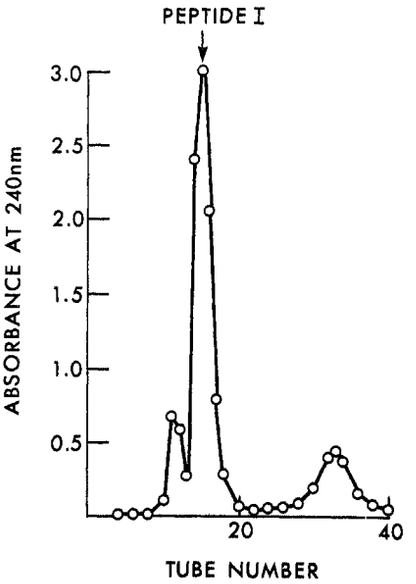

Figure 1.—Carboxymethylcellulose chromatography of partially purified peptide I.

peptides. We therefore prepared N^{α}-acetyl-*S*-(3,4-dimethylbenzyl)cysteinamide[6] and subjected it to the same treatment with trifluoroacetic acid and found that only 0.2% of the sulfhydryl protection was lost. When the compound was treated with hydrogen fluoride for 10 min at 0° the protecting group was completely removed. The suitability of 3,4-dimethylbenzyl protection for cysteine was then demonstrated by a second synthesis of I with use of N^{α}-Boc-*S*-(3,4-dimethylbenzyl)cysteine. Cyclic dodecapeptide I was obtained in highly purified form and was in complete identity with the preparation described above.

For synthesis of the linear carbamidomethyl dodecapeptide II, the carbamidomethylcysteine residues were introduced by use of the *p*-nitrophenyl ester of N^{α}-Boc-*S*-carbamidomethylcysteine. Active ester coupling was selected rather than coupling with dicyclohexylcarbodiimide to avoid any possibility of nitrile formation.[19] With this exception, synthesis of II (Chart II) was carried out in the same manner as the synthesis of I. Deprotection of the linear dicarbamidomethyl dodecapeptide and cleavage from the resin was again achieved with hydrogen fluoride.[13] Purification by gel filtration on Sephadex G-25, partition chromatography[20] on Sephadex G-25 (Figure 2), and chromatography on CMC gave II (~29% yield based on starting Boc-Phe resin) in highly purified form as judged by thin layer and paper chromatography, paper electrophoresis, and amino acid analyses.

Earlier work in this laboratory has shown that the disulfide bridges of HGH can be reduced with dithrothreitol and alkylated with iodoacetamide.[7] We decided to carry out these same reactions with peptide I which should then lead to II and serve as an additional check on our synthetic procedures. When I was so treated the resulting product, after purification by gel filtration on Sephadex G-10 and chromatography on CMC, was found to be identical with II synthesized with *S*-carbamidomethylcysteine.

(19) D. T. Gish, P. G. Katsoyannis, G. P. Hess, and R. J. Stedman, *J. Amer. Chem. Soc.*, **78**, 5954 (1956); C. Ressler, *ibid.*, **78**, 5956 (1956); M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 5688 (1959).

(20) D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **88**, 1310 (1966).

Experimental Section

Melting points were determined on a Fisher-Johns block and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Thin layer chromatography (tlc) was run on silica gel in the following solvents: chloroform–methanol, 1:1 (CM); chloroform–methanol–acetic acid, 15:2:1 (CMA); 1-butanol–acetic acid–water, 4:1:1 (BAW); 1-butanol–pyridine–acetic acid–water 30:20:6:24 (BPAW). Thin layer data cited refer to single-spot chromatograms. Paper chromatography was carried out on Whatman No. 1. Carboxymethylcellulose chromatography was performed in a 1.0 × 55 cm column with a buffer of 0.01 *M* ammonium acetate of pH 4.5. Peaks were detected spectrophotometrically at 240 nm. Styrene–divinylbenzene resin (Bio-Beads S-X1, 200–400 mesh, Bio-Rad Laboratories) served as starting material for solid-phase synthesis, and attachment of Boc-phenylalanine was performed by the standard triethylamine procedure in absolute ethanol.

N^{α}-Boc-*S*-Carbamidomethylcysteine.—*S*-Carbamidomethylcysteine[21] (25.0 g, 140.5 mmol) was converted to the N^{α}-*tert*-butyloxycarbonyl derivative by the procedure of Schnabel[22] with Boc-azide (~214 mmol) at 45° for 10 hr with addition of 4 *N* NaOH to maintain the pH between 9.4 and 9.8. Dioxane was removed *in vacuo;* the solution was diluted with water (300 ml) and washed with three 75-ml portions of ether. The aqueous phase was cooled in ice and acidified to pH 3 with 3 *N* HCl. The product was extracted into ethyl acetate (350 ml), washed with two 50-ml portions of saturated NaCl, and dried over anhydrous $MgSO_4$. Removal of the drying agent and solvent gave an oil which crystallized on standing: yield, 17.4 g. For analysis, a sample (1.40 g) was recrystallized from ethyl acetate (30 ml): 1.24 g, mp 128–131°, tlc (CMA) R_f 0.3, $[\alpha]^{24}D$ −15.2° (*c* 2.00, absolute ethanol).

Anal. Calcd for $C_{10}H_{18}N_2O_5S$ (278.34): C, 43.15; H, 6.52; N, 10.07. Found: C, 43.33; H, 6.42; N, 9.94.

***p*-Nitrophenyl N^{α}-Boc-*S*-Carbamidomethylcysteinate.**—N^{α}-Boc-*S*-carbamidomethylcysteine (5.0 g, 18 mmol) and *p*-nitrophenol (10.0 g, 72 mmol) were dissolved in 15 ml of DMF and cooled to −10°. At 4°, over a period of 30 min, DCC (4.11 g, 19.5 mmol) dissolved in 5 ml of DMF was added. After 18 hr at 4° and 7 hr at 24°, the mixture was filtered at 4° and the solid washed with 50 ml of DMF. Removal of solvent in the combined filtrate and washings gave an oil which crystallized from ether: yield, 6.2 g; mp 145°. Recrystallization from DMF–water gave 5.36 g, mp 161–162°. Further recrystallization of 1.0 g from ethyl acetate gave 0.83 g, mp 162–164°, tlc (BAW) R_f 0.65, $[\alpha]^{24}D$ −41.5° (*c* 2.01, DMF).

Anal. Calcd for $C_{16}H_{21}N_3O_7S$ (399.42): C, 48.11; H, 5.30; N, 10.52. Found: C, 48.04; H, 5.57; N, 10.49.

γ-*p*-Bromobenzyl Glutamate.—To a stirred solution of 51.5 g of sodium copper glutamate[23] in 426 ml of water was added a solution of 64.0 g of *p*-bromobenzyl bromide in 853 ml of DMF. After the mixture stirred for 24 hr at 35–40°, the product was precipitated by addition of 1.3 l. of acetone and then filtered and washed with 200-ml portions of water and acetone. The material (~75 g) was suspended in 5.5 l. of water, 75 g of (ethylenedinitrilo)tetraacetic acid disodium salt was added, and the mixture was boiled until a solution was obtained. The product which came out of solution upon cooling was filtered and washed with 200-ml portions of water and acetone: yield, 25 g (32%); mp 180–181°; tlc (BAW) R_f 0.62; $[\alpha]^{24}D$ +1.4° (*c* 1, 80% acetic acid).

Anal. Calcd for $C_{12}H_{14}NO_4Br$ (316.15): C, 45.59; H, 4.46; N, 4.43. Found: C, 45.64; H, 4.01; N, 4.39.

N^{α}-Boc-γ-(*p*-Bromobenzyl)glutamic Acid.—To a stirred suspension of 18.5 g γ-*p*-bromobenzyl glutamate in 250 ml of DMSO, 16.0 ml of triethylamine (117 mmol) and 13.5 ml of Boc-azide (87.75 mmol) were added.[24] The mixture was stirred for 23 hr at 24°. The solution was diluted with water (840 ml) and washed with two 150-ml portions of ether. The aqueous phase was then cooled and acidified with 50 ml of 3 *N* HCl to pH 2. The product was extracted with two 200-ml portions and one 100-ml portion of ethyl acetate. The ethyl acetate solution was washed with three 50-ml portions of water and then dried over anhydrous $MgSO_4$. Removal of the drying agent and solvent gave an oil which crystallized from ether–petroleum ether (bp 30–60°) in a cold room (4°) overnight. The product was filtered and washed with ether–petroleum ether (1:4): yield, 19.3 g (46.4 mmol, 79%); mp 91–92°; tlc (CM) R_f 0.69; $[\alpha]^{24}D$ −10.7° (*c* 2.01, DMF).

Anal. Calcd for $C_{17}H_{22}NO_6Br$ (416.27): C, 49.05; H, 5.33; N, 3.37. Found: C, 49.26; H, 5.16; N, 3.17.

3,4-Dimethylbenzylcysteine.—A mixture of cysteine free base (29 g, 240 mmol), ammonium chloride (13 g, 243 mmol), and water (1 ml) was stirred in 900 ml of liquid ammonia at the boiling point while 3,4-dimethylbenzyl chloride (55.5 g, 0.36 mol) was added over a period of 1 hr. The mixture was allowed to stir and evaporate to air overnight. The solid residue was dissolved in 850 ml of 1 *N* NaOH and washed with two 500-ml portions of ether. The aqueous solution was cooled in ice and the pH was adjusted to 7 with concentrated HCl. After 4 hr at 4° the solid was collected and washed with 1 l. of cold water. This material was boiled in 3.5 l. of water and filtered while hot. The filtrate was stored at 4° overnight. The product was collected and washed with cold water and acetone: yield, 26.1 g (46% yield); mp 193–196°. For analysis a sample was recrystallized twice from hot water: mp 195–197°, tlc (BAW) R_f 0.57, $[\alpha]^{24}D$ −15.7° (*c* 2, 80% acetic acid).

Anal. Calcd for $C_{12}H_{17}NO_2S$ (239.34): C, 60.22; H, 7.16; N, 5.85. Found: C, 60.45; H, 7.43; N, 5.95.

N^{α}-Boc-*S*-(3,4-Dimethylbenzyl)cysteine Dicyclohexylamine Salt.—3,4-Dimethylbenzylcysteine (6.0 g, 25 mmol) in 60 ml of 67% aqueous dioxane was treated over a 3-hr period with Boc-azide (7.0 ml, 50 mmol) at pH 9–9.5 at 45° with addition of 4 *N* NaOH. The solution was evaporated *in vacuo* to remove dioxane, diluted with water (60 ml), washed with two 40-ml portions of ether, and acidified with cooling to below pH 3 with 3 *N* HCl. The product was extracted with ethyl acetate (75 ml). The ethyl acetate solution was washed with three 25-ml portions of water and dried over anhydrous $MgSO_4$. Removal of the drying agent and solvent gave 8.9 g of oil, tlc (CM) R_f 0.65. The dicyclohexylamine salt was formed in ether by addition of 5.0 ml of dicyclohexylamine with cooling. Crystalline product was obtained from ether–petroleum ether: yield, 11.6 g. Two recrystallizations from chloroform–petroleum ether gave 8.8 g of the salt: mp 122–124°, $[\alpha]^{24}D$ −20.0° (*c* 2.25, 80% acetic acid).

Anal. Calcd for $C_{29}H_{48}N_2O_4S$ (520.78): C, 66.75; H, 9.47; N, 5.37. Found: C, 66.88; H, 9.34; N, 5.39.

For use in synthesis, 5.2 g (10 mmol) of salt was stirred at 4° with ethyl acetate (50 ml) and 1 *N* H_2SO_4 (11 ml). The ethyl acetate layer was washed with four 10-ml portions of cold water and then dried at room temperature over anhydrous $MgSO_4$. Removal of drying agent and solvent gave 3.7 g of oil, tlc (CM) R_f 0.63.

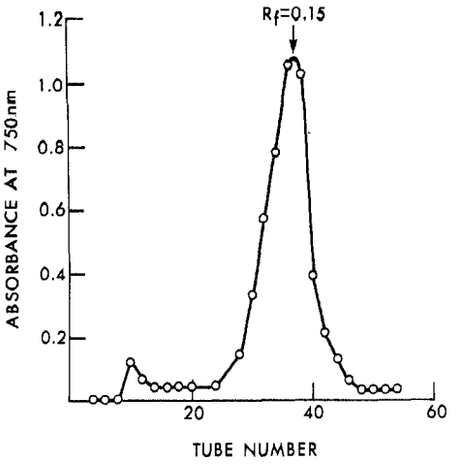

Figure 2.—Partition chromatography on Sephadex G-25 of partially purified peptide II.

Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe (I). A. With N^{α}-Boc-*S*-(*p*-Methoxybenzyl)cysteine.—Boc-Phenylalanyl resin (1.00 g, 0.43 mmol of phenylalanine) was treated by the following schedule of synthesis at 24° for the introduction of each

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(22) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).

(23) R. Ledger and F. H. C. Stewart, *Australian J. Chem.*, **18**, 1477 (1965).

(24) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman and Co., San Francisco, Calif., 1969, p 29.

residue: (1) 2 × 35 ml of CH_2Cl_2, retention volume of resin 7 ml; (2) 35 ml of 50% (v/v) TFA–CH_2Cl_2, 1 min; (3) 35 ml of 50% (v/v) TFA–CH_2Cl_2, 10 min; (4) 3 × 35 ml of CH_2Cl_2; (5) 3 × 35 ml of 50% EtOH–$CHCl_3$; (6) 3 × 35 ml of $CHCl_3$; (7) 35 ml of 3% (v/v) diisopropylethylamine in $CHCl_3$, 5 min; (8) 3 × 35 ml of $CHCl_3$; (9) 3 × 35 ml of CH_2Cl_2; (10) 1.72 mmol (4 equiv) of the appropriate Boc-amino acid in 15 ml of CH_2Cl_2, 10 min; (11) 1.71 mmol of dicyclohexylcarbodiimide in 4.10 ml of CH_2Cl_2, 2 hr; (12) 2 × 35 ml of CH_2Cl_2; (13) 3 × 35 ml of EtOH; (14) 3 × 35 ml of CH_2Cl_2. Side-chain protecting groups employed were benzyl for serine, *p*-bromobenzyl for glutamic acid, tosyl for arginine, and *p*-methoxybenzyl for cysteine. For introduction of the glutamine residue, the three washings in step 9 were replaced by DMF, Boc-glutamine *p*-nitrophenyl ester (4.29 mmol) in 15 ml of DMF was added in step 10 followed by an 18-hr coupling time, and step 11 was replaced with washings of two 35-ml portions of DMF. The valine residues were incorporated by employing 2.58 mmol (6 equiv) of N^{α}-Boc-valine in 15 ml of dichloromethane in step 10 and by addition of 2.57 mmol of DCC in 6.15 ml of dichloromethane in step 11; for the arginine residue, N^{α}-Boc-N^{G}-tosylarginine was dissolved in 16.5 ml of 10% DMF in dichloromethane for step 10, and a 4-hr coupling time was used in step 11. Final weight of finished protected peptide resin was 1.63 g.

A portion (761 mg) of resin was treated with HF (15 ml) for 1 hr at 0° in the presence of anisole (1.0 ml). After removal of the HF, the resin was dried *in vacuo* and then stirred with TFA (15 ml) for 15 min and filtered. The filtrate was evaporated *in vacuo*, and the oily residue was stirred with 75 ml of 0.18 *N* acetic acid and washed with two 25-ml portions of ethyl acetate. The aqueous phase was made up to 500 ml with water. The Ellman test[25] for sulfhydryl content on an aliquot indicated 190 μmol of sulfhydryl groups to be present in the total crude product. The pH was adjusted to 8.1 with 1 *N* NH_4OH (~14 ml), and over a period of 30 min with stirring 0.01 *N* $K_3Fe(CN)_6$ (20.0 ml) was added. The Ellman test on an aliquot taken 15 min after the end of the titration indicated ~6.7 μmol of sulfhydryl groups remaining. To the solution, 20 ml (wet volume) of AG3-X4A resin (chloride form) (Bio-Rad Laboratories, Richmond, Calif.) was added and, after stirring for 15 min, the mixture was filtered and the filtrate lyophilized. The resulting material was subjected to gel filtration on a 2.5 × 133 cm Sephadex G-25 column in 0.5 *N* acetic acid. The peptide material in the major peak (elution volume 472 ml) as detected spectrophotometrically at 240 nm was isolated (93 mg) and submitted to chromatography on CMC (Figure 1). A major peak was detected (elution volume 60–80 ml) and isolation by lyophilization gave 66.5 mg of cyclic dodecapeptide I (~26% yield based on starting resin): tlc (BPAW) R_f 0.28; paper chromatography (BPAW) R_f 0.25; $[\alpha]^{24}D$ −34.9° (*c* 0.62, 0.1 *N* acetic acid).

Paper electrophoresis in pyridine–acetate buffer (pH 3.7, 400 V, 4 hr) showed one ninhydrin-positive, chlorine-positive spot at R_f 0.31 (with respect to lysine). Paper electrophoresis in collidine–acetate buffer (pH 6.9, 400 V, 4 hr) showed one ninhydrin-positive, chlorine-positive spot at R_f 0.10. Amino acid analysis of an acid hydrolysate gave $Gly_{2.09}Glu_{1.99}Val_{1.98}Ser_{1.77}Arg_{0.96}Phe_{0.99}$ and half-cystine$_{1.96}$.

The monomeric nature of I was established by the partial dinitrophenylation method[17] in which a monomer of I should give two products while a dimer of I would give three products. A sample of I (1 mg) was dissolved in 100 μl of 80% aqueous DMSO and treated with 5 μl of 0.1 *M* 2,4-dinitrofluorobenzene in 75% aqueous dioxane for 4 min at 24°. Paper electrophoresis (pH 2.1, 2 kV, 1 hr) gave two spots: one with R_f 0.37 (ninhydrin and Pauly positive) relative to lysine corresponding to I and the second at the origin (yellow, Pauly positive and ninhydrin negative). Further dinitrophenylation of I gave a decreased amount of unreacted I and an increase in the second spot with no appearance of a third spot.

B. With N^{α}-Boc-*S*-(3,4-dimethylbenzyl)cysteine.—Boc-phenylalanyl resin (0.97 g, 0.415 mmol of phenylalanine) was placed in a Beckman Model 990 peptide synthesizer and submitted to the same schedule as in the aforementioned synthesis except that chloroform was replaced by dichloromethane. The side-chain protecting groups employed were also the same as in the aforementioned synthesis with the exception that 3,4-dimethylbenzyl protection for cysteine was employed. Final weight of finished protected peptide resin was 1.50 g.

A portion (682 mg) of resin was treated with HF (15 ml) for 1 hr at 0° in the presence of anisole (1.0 ml). The crude product was worked up as above. The sulfhydryl group content before and after oxidation with 0.01 *N* $K_3Fe(CN)_6$ were 170 and 4.5 μmol, respectively. The major peptide material after the gel filtration on Sephadex G-25 as above (elution volume 465 ml) was isolated (71.0 mg). An aliquot (36.5 mg) of the product was then submitted to the chromatography on CMC as described above. A major peak was detected (elution volume 52–72 ml), and isolation by lyophilization gave 24.8 mg (~20% yield based on starting resin) of cyclic dodecapeptide I, $[\alpha]^{24}D$ −33.1° (*c* 0.60, 0.1 *N* acetic acid). This material was identical to the preparation described in part A on paper electrophoresis, tlc (BPAW), and paper chromatography. Amino acid analysis of an acid hydrolysate gave $Gly_{2.00}Glu_{1.97}Val_{1.04}Ser_{1.81}Arg_{0.93}Phe_{1.03}$ and half-cystine$_{1.87}$.

Val-Gln-Cys(CH_2CONH_2)-Arg-Ser-Val-Glu-Gly-Ser-Cys(CH_2CONH_2)-Gly-Phe (II).—Boc-Phenylalanyl resin (1.00 g, 0.43 mmol of phenylalanine) was carried through the same cycle of steps as described above for the synthesis employing *p*-methoxybenzylcysteine with the following exceptions for the introduction of the *S*-carbamidomethylcysteine residues: the three washings in step 9 were replaced by DMF; 3.86 mmol (9 equiv) N^{α}-Boc-*S*-carbamidomethylcysteine *p*-nitrophenyl ester in 15 ml of DMF was added in step 10 followed by an 18-hr coupling time; step 11 was replaced by washing with two 35-ml portions of DMF. The other side-chain protecting groups employed were the same as in the aforementioned syntheses. Final weight of finished protected peptide resin was 1.52 g.

A portion (760 mg) of resin was treated with HF (15 ml) for 1 hr at 0° in the presence of anisole (1.0 ml). After removal of the HF, the resin was dried *in vacuo* and then stirred with TFA (15 ml) for 15 min and filtered. The filtrate was evaporated *in vacuo*, and the oily residue dissolved in 10 ml of 0.5 *N* acetic acid and washed with two 10-ml portions of ether. The aqueous solution was lyophilized and the resulting material was subjected to gel filtration on Sephadex G-25 as described above and the peptide material in the major peak (elution volume 446 ml) was isolated (167 mg). An aliquot (52.2 mg) was then subjected to partition chromatography on Sephadex G-25 (1.92 × 69 cm) in the solvent system 1-butanol–pyridine–0.2 *N* aqueous NH_4OH containing 0.1% acetic acid (5:3:12). As determined by Folin–Lowry analysis[26] a single symmetrical peak with R_f 0.15 was obtained (Figure 2). The isolated material (40.9 mg) was then submitted to the chromatography on CMC. One peak was detected (elution volume 48–63 ml) and isolation by lyophilization gave 27.1 mg (~29% yield based on starting resin) of linear dodecapeptide II: tlc (BPAW) R_f 0.22, paper chromatography (BPAW) R_f 0.10, $[\alpha]^{24}D$ −49.3° (*c* 0.46, 0.1 *N* acetic acid).

On paper electrophoresis at pH 3.7 and at pH 6.9 peptide II migrated as a single spot at the same rate as peptide I. Amino acid analysis of an acid hydrolysate gave $Gly_{2.00}Glu_{2.05}Val_{2.12}Cys(CH_2COOH)_{2.06}Ser_{1.80}Arg_{1.00}Phe_{1.03}$. Amino acid analysis of a 24-hr leucine aminopeptidase digest gave $Gly_{2.00}Glu_{1.00}(Ser + Gln)_{2.80}Val_{2.10}Cys(CH_2CONH_2)_{0.82}Arg_{0.96}Phe_{0.99}$. When an authentic sample of carbamidomethylcysteine is treated under conditions for enzyme digest, ~60% of the compound disappears as judged by amino acid analysis and quantitatively accounts for the low value obtained on II.

Conversion of I to II.—A sample (30.8 mg) of I was dissolved in 0.01 *M* NH_4HCO_3 (6.0 ml) of pH 8.3 under conditions described previously.[27] Reduction with dithiothreitol[28] (37.6 mg) was followed by treatment with iodoacetamide (449.9 mg). The solution was subjected to gel filtration on Sephadex G-10 (2.2 × 25 cm) in 0.01 *M* NH_4HCO_3 of pH 8.3. The peptide material (elution volume of peak, 48 ml) was isolated (35.8 mg) and submitted to chromatography on CMC. One peak was detected in the position expected for II (elution volume 49–65 ml) and isolation by lyophilization gave 24.8 mg (~74%): tlc (BPAW) R_f 0.22 and paper chromatography (BPAW) R_f 0.10 identical with those of an authentic sample of II; $[\alpha]^{24}D$ −50.7° (*c* 0.402, 0.1 *N* acetic acid). Amino acid analyses of an acid hydrolysate and a leucine aminopeptidase digest gave $Gly_{2.00}Glu_{2.05}Val_{1.99}Cys(CH_2COOH)_{2.19}Ser_{1.93}Arg_{0.95}Phe_{1.02}$ and $Gly_{2.00}Glu_{0.97}(Ser + Gln)_{2.95}Val_{1.90}Cys(CH_2CONH_2)_{0.93}Arg_{1.02}Phe_{0.99}$, respectively.

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Registry No.—I, 41174-26-9; II, 41117-58-2; N^{α}-Boc-*S*-carbamidomethylcysteine, 41117-59-3; *S*-carbamidomethylcysteine, 17528-66-4; *p*-nitrophenyl N^{α}-Boc-*S*-carbamidomethylcysteinate, 41117-61-7; γ-*p*-bromobenzyl glutamate, 20806-21-7; *p*-bromobenzyl bromide, 589-15-1; N^{α}-Boc-γ-(*p*-bromobenzyl)-glutamic acid, 41117-62-8; triethylamine, 121-44-8; Boc-azide, 1070-19-5; 3,4-dimethylbenzylcysteine, 41594-21-2; cysteine, 52-90-4; ammonium chloride, 12125-02-9; 3,4-dimethylbenzyl chloride, 102-46-5; N^{α}-Boc-*S*-(3,4-dimethylbenzyl)cysteinedicyclohexylamine salt, 41117-64-0; N^{α}-Boc-*S*-(*p*-methoxybenzyl)-cysteine, 18942-46-6; N^{α}-Boc-*S*-(3,4-dimethylbenzyl)cysteine, 41117-66-2; human pituitary growth hormone, 9002-72-6.

o-Nitrophenyl Esters of *tert*-Butyloxycarbonylamino Acids and Their Application in the Stepwise Synthesis of Peptide Chains by a New Technique[1]

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Synthesis of the protected nonapeptide *tert*-butyloxycarbonyl-L-leucyl-L-glutaminyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide, corresponding to the C-terminal sequence of a secretin analog, is described. Acylation of L-valinamide with the *o*-nitrophenyl ester of *tert*-butyloxycarbonyl-L-leucine yielded a dipeptide derivative which, after deprotection with trifluoroacetic acid, was converted to a protected tripeptide amide by reaction with *tert*-butyloxycarbonylglycine *o*-nitrophenyl ester. The chain was lengthened in the same manner; *o*-nitrophenyl esters of Boc-amino acids were used as acylating agents. All the operations were carried out in the same vessel, from which the intermediates were not removed throughout the synthesis. The preparation and properties of *o*-nitrophenyl esters of Boc-amino acids are also reported.

Stepwise synthesis of peptide chains was proposed, and demonstrated on the example of oxytocin, by Bodanszky and du Vigneaud.[2] This approach proceeds through isolated intermediates. If unequivocal methods are used in the acylation and deprotection reactions, it permits rapid chain lengthening, since an extensive purification of the intermediates is often unnecessary. Removal of excess reagents and of by-products was accomplished, in the majority of steps, simply by washing with appropriately chosen solvents. Active esters, such as *p*-nitrophenyl esters, were found as the acylating agents of choice in this method. The repetitiveness of the operation led to the suggestion[3] that mechanization and automation of the procedure would eventually be possible. In the following years, the stepwise strategy indeed lent itself to the development of mechanized and automated syntheses of long chains, but only through the introduction of the solid-phase method by Merrifield.[4] Without a solid support, peptides of only moderate size[5-7] were built by the stepwise approach. In this paper we propose a new technique by which the stepwise synthesis of peptides, *not attached to an insoluble polymer*, can be considerably facilitated.

tert-Butyloxycarbonyl-L-valinamide was deprotected with trifluoroacetic acid, the reagent was removed *in vacuo*, and the residue was triturated with dry ether. These operations were carried out in a centrifuge tube provided with a standard tapered joint through which the tube could be attached to a rotary evaporator. After separation of the trifluoroacetate salt of L-valinamide from the ethereal solution by centrifugation, the supernatant was removed, and the precipitate was washed with ether and dried *in vacuo*. After dissolution of the trifluoroacetate salt in dimethylformamide and the addition of a tertiary amine,[8] the amino component was acylated with an active ester. *o*-Nitrophenyl esters of protected amino acids, reagents that remain efficient even under hindered conditions,[9,10] *e.g.*, in solid-phase synthesis,[11] performed quite satisfactorily in this procedure. High concentration of the reactants is desirable for practical rates and for the suppression of intramolecular side reactions.[12] Therefore, with the growing chain length, the *o*-nitrophenyl esters were applied in gradually increasing excess, their concentration at the start of each acylation being not less than 0.1 *M*. Completion of the acylation was ascertained by a spot test with ninhydrin. The amount of the released *o*-nitrophenol also is a good measure of the extent of acylation. Evaporation of the solvent was followed by the addition of a "nonsolvent." Ethyl acetate is particularly useful in this role[13] because it precipitates many of the common protected peptide intermediates while keeping in solution the excess active esters, the released *o*-nitrophenol and—importantly—also the trifluoroacetate salts of triethyl-

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