mg of LiAlH in 50 ml of dry ether at room temperature. After stirring overnight, the reaction was cautiously quenched with wet ether. The separated aluminate was filtered and washed generously with ether. The organic filtrate was washed with water, dried, and evaporated to give a mobile yellow oil. Distillation (110–115° (0.05 mm)) gave 680 mg of colorless oil: nmr δ 2.20 (s, 3 H), 2.80 (s, 4 H), 3.75 (s, 3 H), 3.80 (s, 3 H), 6.53 (s, 1 H), 6.60 (s, 1 H), 7.17 (s, 5 H); mass spectrum *m/e* 256 (13), 165 (100), and 91 (39).

Anal. Calcd for $C_{17}H_{20}O_2$: C, 79.65; H, 7.86. Found: C, 79.41; H, 8.11.

Somatostatin. Total Solid Phase Synthesis

Jean E. F. Rivier

Contribution from the Salk Institute for Biological Studies, La Jolla, California 92037. *Received November 5, 1973*

Abstract: The structure of a hypothalamic somatotropin-release inhibiting factor (SRIF, somatostatin) of ovine origin, the sequence of which is H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, has been reported. We describe: (a) the synthesis on a chloromethylated resin of the corresponding linear peptide; (b) its deprotection and cleavage from the resin by HF; (c) its purification by repeated gel filtrations in presence of β-mercaptoethanol; (d) oxidation of the sulfhydryls of both cysteinyl residues to form the 38-membered ring; (e) characterization of the straight-chain intermediate and bridged tetradecapeptide after purification; (f) comparison of natural somatostatin with its synthetic counterpart by tlc in three different systems, electrophoresis on paper, amino acid analysis after hydrolysis under various conditions, optical rotation, circular dichroism, gel filtration and partition chromatography on Sephadex G-25F, tlc of the tryptic digest, mass spectrometry of the derivatized fragment -Asn-Phe-Phe-Trp-Lys- isolated from a tryptic digest, and biological activity.

The tetradecapeptide somatostatin,[1] H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, was isolated from ovine hypothalamic extracts[2] on the basis of its ability to inhibit the secretion of radioimmunoassayable growth hormone (GH) by primary cultures of enzymatically dispersed rat anterior pituitary cells. The sequence was obtained using the regular Edman degradation procedure[3] coupled to mass spectrometric analysis of a tryptic digest.[4] Extensive biological studies made on a synthetic material, the synthesis of which has already been described in a preliminary note,[5a] have been reported.[6–8] It is noteworthy that somatostatin has been found to inhibit in normal human subjects the release of growth hormone brought about by L-Dopa, infusion of arginine,[9,10] and in diabetics, by exercise;[11] it is highly active in lowering plasma levels of GH in acromegalics[10] and thus, as originally proposed,[1] may be of therapeutic value in acromegaly and diabetes. We report here in detail the first synthesis[5a] of somatostatin in a highly purified form and results from the comparison of the physical and biological properties of both synthetic and natural peptides. This comparison should not be considered as a proof of reported structure[1,3,4] by synthesis but as another means of identification of the synthetic material made by the still controversial solid phase methodology, the drawbacks and advantages of which are discussed for this particular peptide.

The protected somatostatin tetradecapeptide was synthesized in a stepwise manner on chloromethylated resin prepared according to Stewart and Young.[12] Boc-Cys(*p*-OMe-Bzl) was esterified by the classical method,[12] even though esterification in DMSO in the presence of potassium *tert*-butoxide according to Monahan and Gilon[13] was also found to be appropriate. For both methods the optical purity of the cysteine attached to the resin was checked by the synthesis of the dipeptide Leu-Cys(Cm). Boc-Leu was coupled to the free Cys(*p*-OMe-Bzl)-polymer: the dipeptide was deprotected and cleaved from the resin in HF[14] and

(1) P. Brazeau, W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier, and R. Guillemin, *Science*, **179**, 77 (1973). We proposed to name the peptide described here somatostatin, from somato(tropin), a pituitary factor affecting statural growth, and stat(in), from the Latin "to halt, to arrest" (as in homeostat or bacteriostat). This is in keeping with the efforts of several international nomenclature committees (with which the final decision should remain) aiming at creating trivial names for biologically active polypeptides rather than maintaining the use of acronyms such as SRIF, somatotropin release inhibiting factor. Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1971). Other abbreviations used are: GH, growth hormone; DMF, dimethylformamide; Z, benzyloxycarbonyl, Z(2 Cl), 2-chlorobenzyloxycarbonyl.

(2) Brazeau, *et al.*, submitted for publication in *Can. J. Biochem.*

(3) R. Burgus, N. Ling, M. Butcher, and R. Guillemin, *Proc. Nat. Acad. Sci. U. S.*, **70**, 684 (1973).

(4) N. Ling, R. Burgus, J. Rivier, W. Vale, and P. Brazeau, *Biochem. Biophys. Res. Commun.*, **50**, 127 (1973).

(5) (a) J. Rivier, P. Brazeau, W. Vale, N. Ling, R. Burgus, C. Gilon, J. Yardley, and R. Guillemin, *C. R. Acad. Sci.*, **276**, 2737 (1973). While this work was in progress three reports of independent syntheses have successively appeared in the literature: (b) D. Sarantakis and W. A. McKinley, *Biochem. Biophys. Res. Commun.*, **54**, 234 (1973); (c) D. Yamashiro and C. H. Li, *Biochem. Biophys. Res. Commun.*, **54**, 882 (1973); (d) D. H. Coy, E. J. Coy, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **54**, 1267 (1973).

(6) W. Vale, P. Brazeau, G. Grant, A. Nussey, R. Burgus, J. Rivier, N. Ling, and R. Guillemin, *C. R. Acad. Sci.*, **275**, 2913 (1972).

(7) W. Vale, P. Brazeau, C. Rivier, J. Rivier, G. Grant, R. Burgus, and R. Guillemin, *Endocrinology*, **93**, A139 (1973).

(8) P. Brazeau, J. Rivier, W. Vale, and R. Guillemin, *Endocrinology*, **94**, 184 (1974).

(9) T. M. Siler, G. VandenBerg, S. S. C. Yen, P. Brazeau, W. Vale, and R. Guillemin, *J. Clin. Endocrinol. Metab.*, **37**, 632 (1973).

(10) R. Hall, A. V. Schally, D. Evered, A. J. Kastin, C. H. Mortimer, W. M. G. Tunbridge, G. M. Besser, D. H. Coy, D. J. Goldie, A. S. McNeilly, C. Phenekos, and D. Weightmen, *Lancet*, **2**, 581 (1973).

(11) Aa. P. Hansen, H. Orskov, K. Seyer-Hansen, and K. Lundbaek, *Brit. Med. J.*, **3**, 523 (1973).

(12) J. Stewart and J. Young, "Solid Phase Peptide Synthesis," W. H. Freeman, San Francisco, Calif., 1969.

(13) M. Monahan and C. Gilon, *Biopolymers*, **12**, 2513 (1973).

(14) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jap.*, **40**, 2164 (1967).

carboxymethylation took place in the usual way.[15] Analysis of the resulting products by the sensitive procedure of Manning and Moore[16] showed only one major peak corresponding to L-Leu-L-Cys(Cm); the maximum amount of L-Leu-D-Cys(Cm) that could have been present was less than 0.1%. Since N^{α}-Boc protection was used throughout the synthesis, a suitable selection of side-chain protecting groups was required. For threonine and serine, O-Bzl protection was chosen. Since Z protection for lysine was reported to be unstable, we used the Z (2-Cl) protecting group which was reported to be 60 times more stable.[17] The active ester of Boc-Asn was used and the coupling carried out in DMF in the usual manner. Boc-Cys(*p*-OMe-Bzl) was used since it is easily removed by HF. Alanine and glycine were introduced as a Z protected dipeptide (Z-Ala-Gly) in order to have a reliable internal standard to evaluate the amino acid analyses. All synthetic steps were carried out manually and as much as 100 g of peptide-resin has been synthesized at one time. The schedule used for the synthesis is reported in the Experimental Section (Tables I and II).

Table I. Schedule for DCC[b] Coupling in Solid Phase Synthesis of Somatostatin

Step	Reagents and operations[a]	Mix times, min
1	CH_2Cl_2 wash, 80 ml (2 times)	3
2	MeOH wash, 30 ml (2 times)	3
3	CH_2Cl_2 wash, 80 ml (3 times)	3
4	50% TFA + 5% 1,2-ethanedithiol in CH_2Cl_2, 70 ml (2 times)	10
5	CH_2Cl_2 wash, 80 ml (2 times)	3
6	Et_3N 12.5% in DMF, 70 ml (2 times)	5
7	MeOH wash, 40 ml (2 times)	2
8	CH_2Cl_2 wash, 80 ml (3 times)	3
9	Boc-AA (10 mmol) in 30 ml of DMF (1 time) + DCC (10 mmol) in DMF	30
10	MeOH wash, 40 ml (2 times)	3
11	Et_3N 12.5% in DMF, 70 ml (1 time)	3
12	MeOH wash, 30 ml (2 times)	3
13	CH_2Cl_2 wash, 80 ml (2 times)	3

Aliquot taken for ninhydrin test: if negative, we go back to step 1, if positive or slightly positive, we go back to step 9 → 13.

[a] AA = amino acid; TFA, F_3CCOOH. [b] DCC, *N,N'*-dicyclohexylcarbodiimide.

Table II. Schedule for Boc-Asn-PNP Coupling in Solid Phase Synthesis of Somatostatin[a]

Step	Reagents and operations	Mix times, min
9	DMF wash, 60 ml (3 times)	3
10	Boc-Asn-PNP (15 mmoles) in 20 ml of DMF (1 time)	800
11	MeOH wash, 30 ml (4 times)	3
12	Et_3N 12.5% in DMF, 30 ml (2 times)	3
13	MeOH wash, 30 ml (2 times)	3
14	CH_2Cl_2 wash, 80 ml (3 times)	3

Aliquot taken for ninhydrin test: if negative we go back to step 1, if positive or slightly positive, we go back to step 9 → 14

[a] Same as lines 1–8 in Table I followed by changes shown.

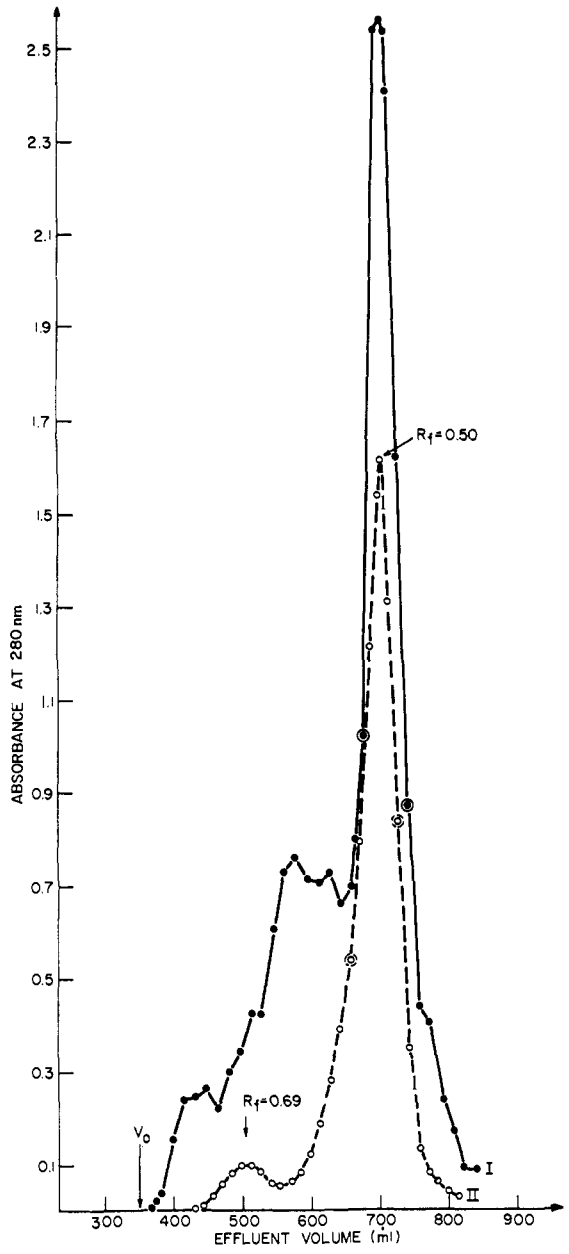

Figure 1. Gel filtration of crude and purified synthetic reduced somatostatin. Column 2.5 × 200 cm Sephadex G-25F, 2 *N* AcOH, 10^{-2} *M* β-mercaptoethanol. V_0 = hold-up volume: profile I (●) 1.25 g crude, yield 670–740 ml, 500 mg, 40%; profile II (○) 500 mg from I, yield 660–728 ml, 350 mg, 28%.

F_3CCOOH was used for cleavage of the Boc protecting groups. 1,2-Ethanedithiol was added for protection of the tryptophan residue from oxidation, a problem that has long been recognized in solid phase synthesis. We are, however, also aware of the suggestion that β-mercaptoethanol[18] may give rise to some sulfenyl halide formations which could lead to the known thioether formation with tryptophan.[19] Coupling time could be reduced to 35 min provided the substitution was lower than 0.15 mequiv/g of resin and a fivefold excess Boc amino acid in DMF in presence of an equimolar amount of *N,N'*-dicyclohexylcarbodi-

(15) C. Anfinsen and E. Haber, *J. Biol. Chem.*, **236**, 1361 (1961).
(16) J. Manning and J. Moore, *J. Biol. Chem.*, **243**, 5591 (1968).
(17) B. Erickson and R. Merrifield, *J. Amer. Chem. Soc.*, **95**, 3757 (1973).
(18) N. Izumiya, M. Waki, T. Kato, M. Ohno, H. Aoyagi, and N. Mitsuyasu, "Chemistry and Biology of Peptides," J. Meienhoffer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 269.
(19) T. Wieland and R. Sarges, *Justus Liebigs Ann. Chem.*, **658**, 181 (1962).

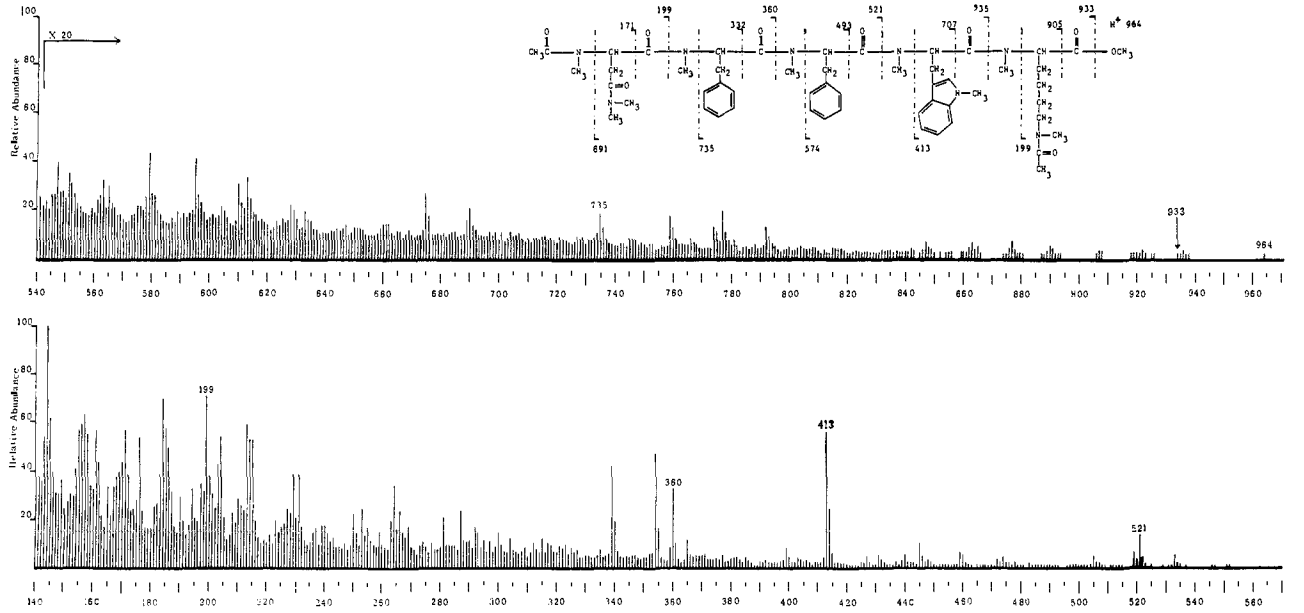

Figure 2. Mass spectrum of a permethylated chloroform extract from an acetylated tryptic digest of natural somatostatin. Probe temperature, 285°.

imide was used. Complete coupling was monitored by the ninhydrin test of Kaiser, *et al.*[20] Under these conditions, all protected amino acids coupled satisfactorily as shown by amino acid analysis of the peptide resin. Cleavage and concomitant deprotection of the tetradecapeptide were achieved by HF in presence of anisole. After elimination of HF under high vacuum and a classical work-up, a white fluffy material that accounted for 60% of the calculated yield was obtained. Amino acid analysis of the cleaved resin revealed the presence of a significant amount of peptide still attached to the resin. Although 25% of the original peptide content was recovered in a second HF treatment, 15% could still be accounted for on the resin. This can best be explained by a relatively poor solubility in the medium (HF–anisole) or a strong interaction with the resin of the cleaved and deprotected peptide which prevents further reaction of HF. The poor effectiveness of HF as a swelling agent could also be involved. The crude material was subjected to gel filtration. Uv absorbance at 280 nm is shown in Figure 1, profile I. The main peak after lyophilization and routine handling was reapplied on the same column under the same conditions in an attempt to obtain one single symmetrical pattern (Figure 1, profile II). Some oxidation had taken place and formation of dimers (polymers) had occurred (see peak R_f 0.69) during lyophilization or upon standing as a dry powder. This was evidenced by the absence of free sulfhydryls measured according to Habeeb's method[21] using Ellman's reagent.[22] Similar observations were made by Ressler[23] while investigating inactivation of oxytocin. A total yield of 28% in purified linear somatostatin was obtained. The relatively large amount of impurities present in the crude peptide is not an uncommon observation for peptides obtained by the solid phase methodology. The reasons are multiple. (a) Failure sequences and chain terminations. They can be reduced to a minimum by using low substituted resin and solvents that properly swell the resin allowing a better access of the reagents to the growing chain.[24] (b) Side chain reactions. These can be partially eliminated when the proper choice of protecting groups is made.[17,25] (c) Side reactions during HF cleavage. Very little can be done although a new very promising replacement reagent has been reported recently by Pless and Bauer.[26]

The major compound (R_f 0.50) was characterized using the following techniques. Amino acid analyses under various hydrolysis conditions (see Experimental Section). We have not investigated the reasons for the consistently lower values (0.5–0.6; theoretical 1.0) obtained for serine after 6 *N* HCl hydrolysis when Cys is under its reduced form as compared with the Ser values when Cys is oxidized (cystine or cysteic acid). The specific optical rotation of the linear tetradecapeptide is $[\alpha]^{22}D$ −38° (*c* 1, 1% AcOH). Sequential analysis of natural (ovine) somatostatin by Edman degradation[3] has been described. A comparative study by mass spectrometry of the derivatized fragment -Asn-Phe-Phe-Trp-Lys- isolated from a tryptic digest of ovine and synthetic linear somatostatin had been reported by Ling *et al.*[4] The two spectra obtained (Figures 2 and 3) are virtually identical. Behavior of the synthetic material during tlc on silica gel and electrophoresis is altered by the presence of the free sulfhydryls of cysteine as shown by Ellman's reagent.[22]

Oxidation Conditions Leading to the Bridged Molecule. Somatostatin. Under neutral and very dilute conditions, and upon standing at room temperature or in the cold in the dark, air brought about cyclization although with relatively poor yield (*ca.* 25%). After lyophilization the powder was partially dissolved in the

(20) E. Kaiser, R. Colescott, C. Bossinger, and P. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(21) A. F. S. A. Habeeb, *Methods Enzymol.*, **25**, 457 (1972).
(22) G. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
(23) C. Ressler, *Science*, **128**, 1281 (1958).
(24) W. S. Hancock, D. J. Prescott, P. R. Vagelos, and G. R. Marshall, *J. Org. Chem.*, **38**, 774 (1973).
(25) B. W. Erickson and R. B. Merrifield, *J. Amer. Chem. Soc.*, **95**, 3750 (1973).
(26) J. Pless and W. Bauer, *Angew. Chem.*, **85**, 142 (1973).

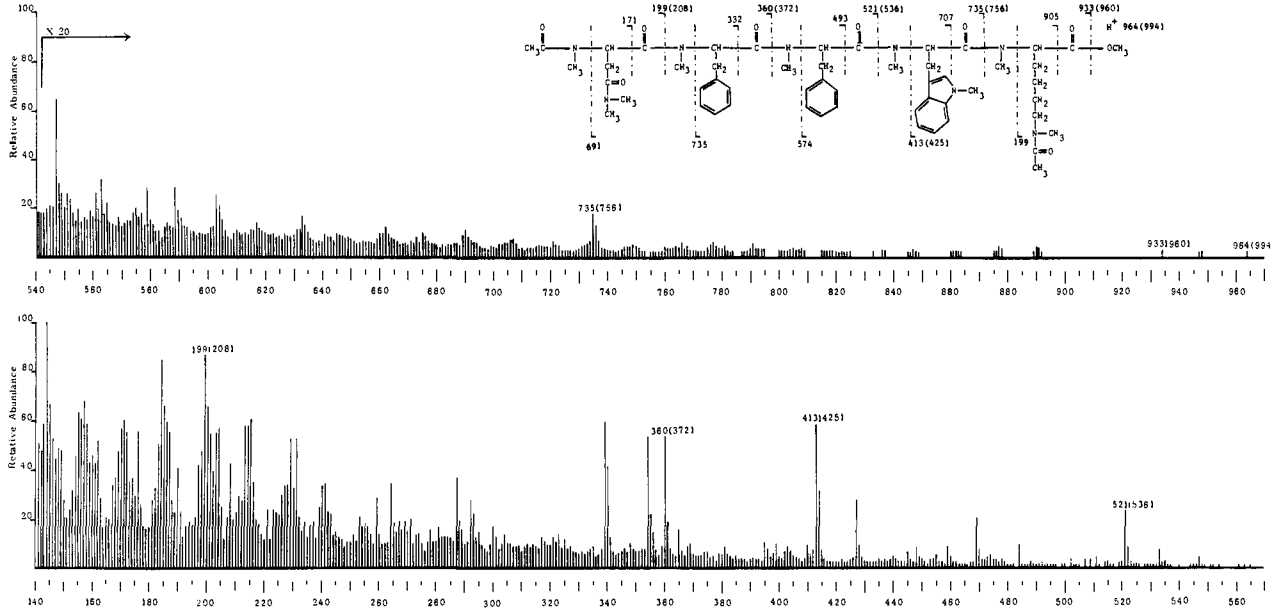

Figure 3. Mass spectrum of a permethylated chloroform extract from an acetylated tryptic digest of synthetic linear somatostatin. Probe temperature, 281°. ( ) indicates peak shift when CH_3I was substituted with CD_3I in the permethylation.

upper phase of a BAW partition system (see Figure 4) and purified on a column yielding the purified synthetic somatostatin after 2.8 V_0.[2] After lyophilization, it was characterized as follows: expected ratios were obtained by amino acid analysis under various hydrolysis conditions as shown in Table III. Cysteine does appear now essentially as cystine after enzymatic or *p*-toluenesulfonic acid hydrolysis; the presence of β-mercaptoethanol used to activate the enzyme apparently formed some mixed disulfide in an exchange reaction with some of the cysteine.[3] Similar results were obtained with the natural compound (Table III). Total oxidation of the cysteinyl sulfhydryl was shown by Habeeb's method[21] using Ellman's reagent.[22]

Table III. Amino Acid Analyses of Ovine and Synthetic Somatostatin

Condition[a]	Asp	2Thr	Ser	Gly	Ala	3Phe	2Lys	NH_3	Trp	2Cys
1	1.0	2.0	0.8	1.0	1.0	2.8	2.0	1.8	0.2	0.8[b]
2	(*ca.* 3.5[d])			1.0	1.0	3.0	1.9		1.1	1.6[c]
3	1.0	1.7	0.8	1.0	0.9	2.8	2.0	2.8		2.0[e]
4	1.0	2.0	0.9	1.0	1.0	2.8	1.8	2.5	0.5	1.2[c]
5	1.0	2.0	0.8	1.0	1.0	2.9	1.9	1.1	0.5	1.7[b]
6	1.1	2.0	1.0	1.1	1.0	2.8	1.9	2.9	0.5	1.5[c]

[a] 1, after 6 *N* HCl containing 0.5% thioglycolic acid, hydrolysis; 2, after enzymatic hydrolysis (F. Sanger, *Biochem. J.*, **44**, 126 (1949)); 3, after oxidation by performic acid and hydrolysis like under 1; 4, after 3 *N* *p*-toluenesulfonic acid containing 0.2% aminoethylindole, hydrolysis; 5, natural somatostatin treated as in 1; 6, natural somatostatin treated as in 4. [b] Appears as half-cystine, other side reaction products being present. [c] Appears as cystine, other side reaction products being present. [d] Asn, Thr, and Ser were not resolved (estimated by average of color values of these amino acids). [e] Appears as cysteic acid.

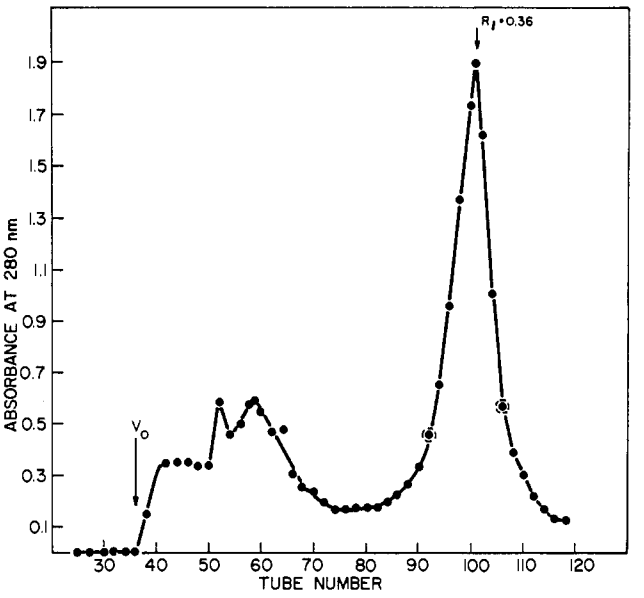

Figure 4. Partition chromatography on Sephadex G-25F of crude cyclized somatostatin. Sample: 160 mg, applied *ca.* 100 mg; insoluble residue *ca.* 60 mg. Fraction size: 1.8 ml. Yields tube 92–106; 40 mg.

Synthetic somatostatin has a specific optical rotation $[\alpha]^{22}D$ $-33.3 \pm 0.5°$ (*c* 1.00, 1% AcOH) and $[\alpha]^{22}D$ $-33.2 \pm 0.5°$ (*c* 1.036, 0.1 *M* AcOH). For the natural peptide, we obtain a specific optical rotation $[\alpha]^{22}D$ $-32.34 \pm 2°$ (*c* 0.244, 1% AcOH). Cyclization of somatostatin by the ferricyanide procedure of du Vigneaud[27] has been recently reported.[5c,d] The yields obtained are, at best, of the same order and the specific optical rotation of the final product as reported varies between $[\alpha]^{24}D$ $-29°$ (*c* 0.5, 0.1 *M* AcOH)[5c] and $[\alpha]^{25}D$ $-39.5°$ (*c* 1.1, 0.1 *M* AcOH).[5d] A product synthesized by the classical approach[5b] and air oxidized according to our procedure gave a product with $[\alpha]^{25}D$ $-36 \pm 1°$ (*c* 0.565, 1% AcOH). Cyclization of somatostatin by the ferricyanide procedure gave us a product identical with that described in this paper. Comparison and characterization of synthetic and natural somatostatin

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

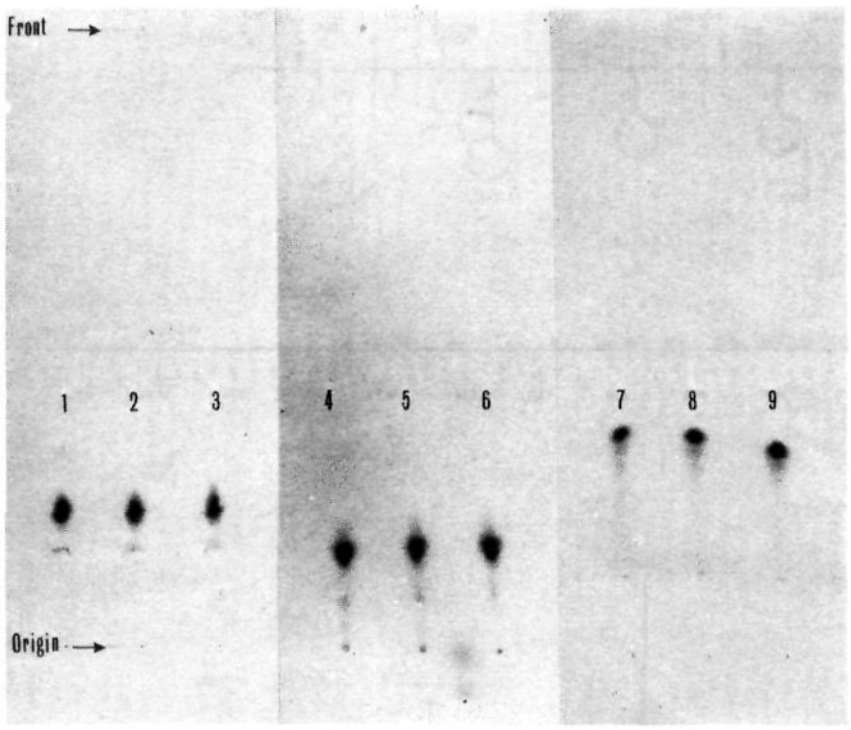

Figure 5. Comparative tlc of natural (10 μg) (1,4,7), natural and synthetic (5 μg each) (2,5,8), and synthetic (10 μg) (3,6,9) somatostatin in three different solvent systems: (1–3) BPyA, *n*-BuOH (5), Pyr (3), 0.1% AcOH (11), R_f (0.33–0.36); (4–6) BIpNEt, *n*-BuOH (1), *i*-PrOH (1), 1 *N* NH_3 (2.5), EtOAc (1), R_f (0.30–0.33); (7–9) BAW; *n*-BuOH (4), AcOH (1), H_2O (5), R_f (0.36–0.40). The visible zones are ninhydrin positive materials. Synthetic material only; not shown. *i*PN; *i*-PrOH (2), 1 *N* NH_3 (1) R_f (0.06–0.14); iPA, *i*-PrOH (2), 1 *N* AcOH (1) R_f (0.83–0.88); iAPyW; *i*-amylOH (7), Pyr (7), H_2O (6), R_f (0.35–0.45); EtPyAW, EtOAc (5), Pyr (5), AcOH (1), H_2O (3), R_f (0.60–0.64).

by tlc are shown in Figure 5. Synthetic somatostatin shows one ninhydrin spot in four additional systems reported in Figure 5. Comparison and characterization of synthetic and natural somatostatin by electrophoresis is shown in Figure 6. The circular dichroism spectra of the synthetic and natural hypothalamic factors are virtually identical as shown in Figure 7. Comparative tlc of a tryptic digest of natural and synthetic somatostatin is shown in three different systems (Figure 8). To exclude the improbable formation of a dimer we applied the synthetic cyclized material on a Sephadex G-25F column; the elution pattern is symmetrical and shown in Figure 9; it is centered at $2.0V_0$. This value is identical with that observed for the natural peptide[2] and the linear peptide after the column had been saturated with *β-mercaptoethanol*. These observations suggest that natural and synthetic cyclic peptides are monomers.

Quantitative *in vitro* bioassays were carried out as described by Vale, *et al.*;[28] they are based on the ability of the products to inhibit the rate of secretion of GH by rat pituitary cells in monolayer tissue cultures. The synthetic preparations (linear and oxidized) of somatostatin in that system are equipotent and equally active in comparison with the natural hormone (see Table IV), in classical 6-point assays with complete statistical validation. Other extensive results, not to be reported here, show that synthetic somatostatin has identical biological activity with that of native (ovine) somatostatin when injected intravenously to laboratory animals.

These results demonstrate that, when used together with the available techniques for purification, the solid phase method of peptide synthesis is the fastest way to synthesize peptides the size of somatostatin, and may become the most economical method for the synthesis of large quantities of peptide.

(28) W. Vale, G. Grant, M. Amoss, R. Blackwell, and R. Guillemin, *Endocrinology*, **91**, 562 (1972).

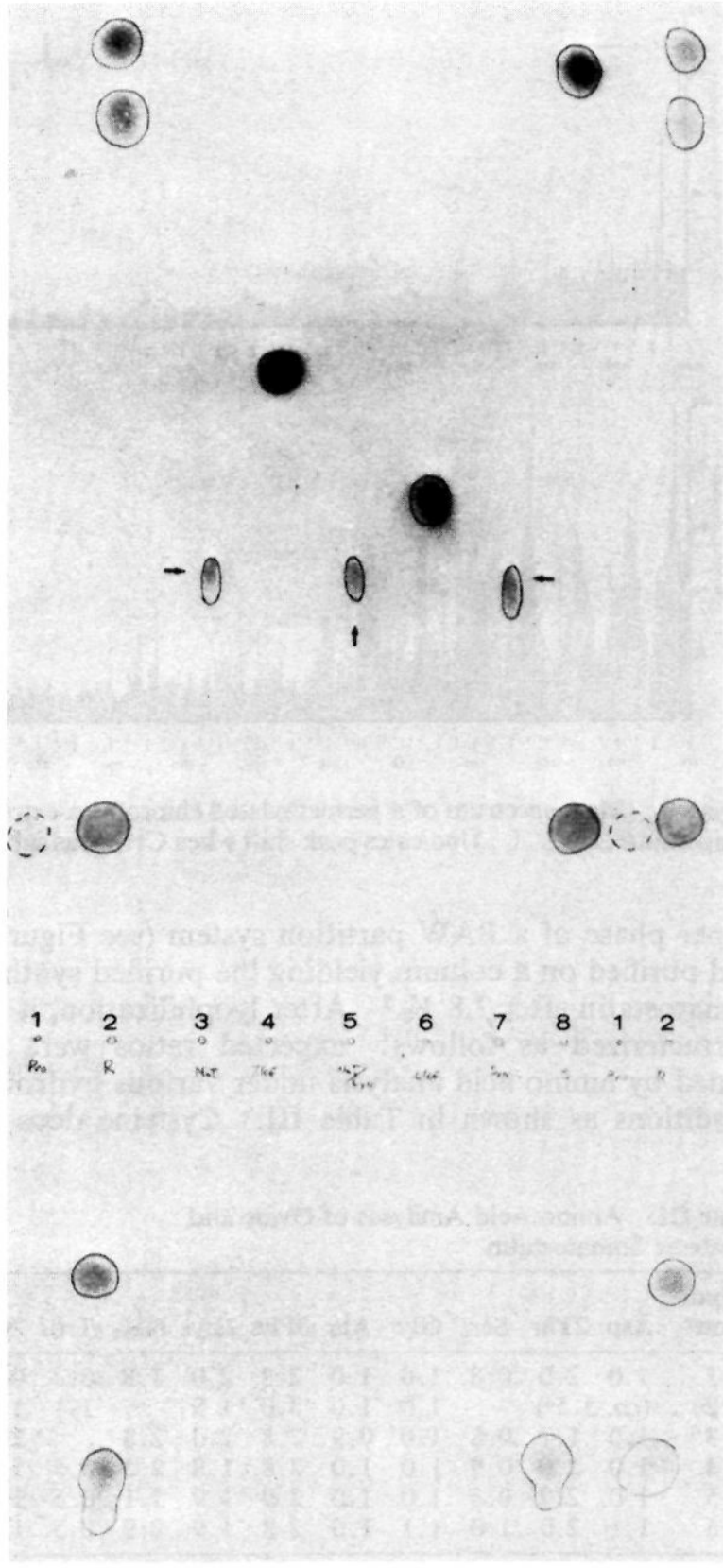

Figure 6. Comparative electrophoresis of natural and synthetic somatostatin. Electrophoresis on paper (Whatman 3MM, pH 4.7, buffer containing 2.5% acetic acid, 2.5% pyridine, 5% *n*-BuOH, and 90% H_2O) at 3500 V, 2.5 hr. The visible zones are ninhydrin and/or Pauly positive materials: 1, proline; 2, references (top to bottom: Lys, Arg, (Leu + Phe + Gly), Glu, Asp; 3 = natural somatostatin (50 μg) R_f 0.33 relative to lysine; 4, pGlu-His-Pro-NH_2 (TRF); 5, mixture of natural (25 μg) and synthetic somatostatin; 6, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LRF); 7, synthetic somatostatin (50 μg); 8, references (top to bottom) His, (Ala + Val + Pro), Asp.

Experimental Section

Amino acid analyses were performed on peptide hydrolysates using a Beckman/Spinco Model 119 amino acid analyzer. Peak areas were determined by an Infotronics Model CRS 100A electronic integrator. Enzymatic hydrolysis was performed as follows: to 100 μg of peptide were added 85 μl of 0.05 *M* NH_4OAc buffer (pH 5.3), 5 μl of 1:32 dilution β-mercaptoethanol in H_2O, and 10 μl of a 0.5 mg/ml of papain (Worthington Biochemical) in

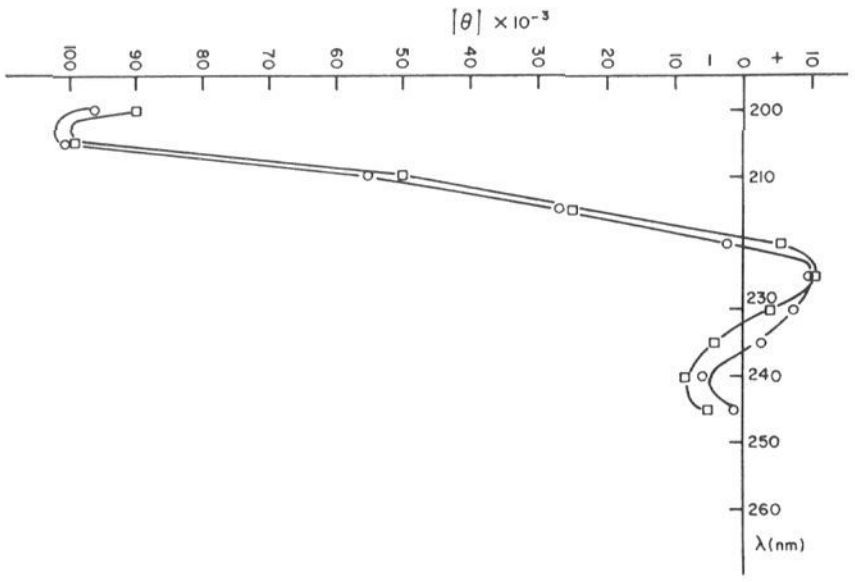

Figure 7. Circular dichroism spectra of natural (○) and synthetic (□) somatostatin.

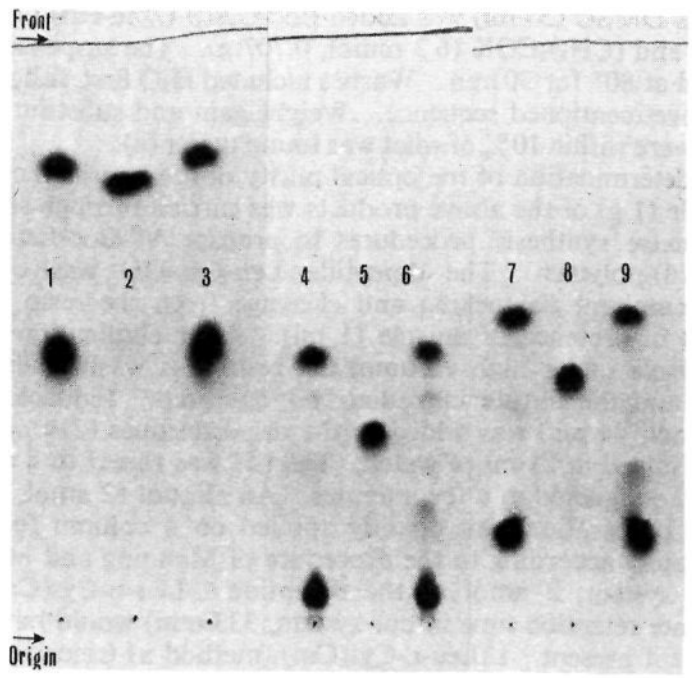

Figure 8. Comparative tlc of a tryptic digest of synthetic (15 μg) (1,4,7) and natural somatostatin (15 μg) (3,6,9), with synthetic somatostatin (15 μg) (2,5,8) as a marker, in three different systems: iPA (1–3), iAPyW (4–6), and BPyA (7–9). Somatostatin and the six spots with the larger R_f (fragment -Asn-Phe-Phe-Trp-Lys-) are uv and ninhydrin positive whereas the six spots with the smaller R_f (peptide H-Ala-Gly-Cys-Lys Thr-Phe-Thr-Ser-Cys-OH) are only ninhydrin positive.

Table IV. Effects of Native, Synthetic, and Reduced Synthetic Somatostatin on the Secretion of GH by Cultured Anterior Pituitary Cells[a]

Treatment	ng of GH secreted/dish over 3 hr ± SEM[b]
Control	660 ± 11
0.2 n*M* native somatostatin	583 ± 62
1.0 n*M* native somatostatin	323 ± 34
5.0 n*M* native somatostatin	226 ± 31
0.2 n*M* synthetic somatostatin	510 ± 66
1.0 n*M* synthetic somatostatin	333 ± 26
5.0 n*M* synthetic somatostatin	246 ± 23
0.2 n*M* reduced synthetic somatostatin	530 ± 50
1.0 n*M* reduced synthetic somatostatin	316 ± 23
5.0 n*M* reduced synthetic somatostatin	256 ± 13
Potencies[c] relative to that of native somatostatin	
Synthetic somatostatin 101% (54–192)[d]	
Reduced synthetic somatostatin 96% (50–179)	

[a] Results taken from: W. Vale, P. Brazeau, C. Rivier, J. Rivier, and R. Guillemin, Proceedings of NIH conference on hGH, Baltimore, Md., Oct 1973; and ref 5a. [b] SEM, standard error of the means. [c] Potencies calculated as multiple 6-point bioassays expressed as % native somatostatin. [d] 95% confidence limits.

H_2O. The solution was incubated for 2 hr at 37°, acidified with 2 drops of AcOH, and lyophilized. To this lyophilized material was added 80 μl of 0.2 *M* $N(CH_3)_3$–AcOH buffer (pH 8.2), 5 μl of 1:32 dilution β-mercaptoethanol in H_2O, and 15 μl of a 10.0 mg/ml of H_2O Amino Peptidase M (Rohm, Inc.). The solution was incubated for 3 hr at 37°, acidified with 2 drops of AcOH, and lyophilized. An aliquot was used for amino acid analysis. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter in 10-cm cells. Concentrations were based on dry weight. CD spectra were recorded on a Cary Model 61 spectropolarimeter. Microanalyses were performed by Galbraith Laboratories, Inc. Thin-layer chromatography (tlc) was run on Eastman 6061 silica gel plates. All solvents were of reagent grade. Highly purified *N,N'*-dicyclohexylcarbodiimide was obtained from Research Plus Laboratories, Inc. All columns used were freshly packed with degassed Sephadex G-25 Fine (Pharmacia Fine Chemicals, Inc.). Columns for gel filtration were packed and equilibrated with 2 *N* CH_3COOH; columns for partition chromatography were packed in 0.5 *N* CH_3COOH then equilibrated with the lower phase of the BAW system (1-butanol–acetic acid–water, 4:1:5) followed by equilibration with the upper phase.[29] The compounds to be purified were loaded onto the columns at the concentration of 100 mg/ml. Absorbance at 280 nm of the collected fractions (dilution, 1:10) was measured with a Beckman DU optics equipped with a Gilford Model 222 photodetector and power supply. Mass spectra were obtained from a Varian CH-5 mass spectrometer with the

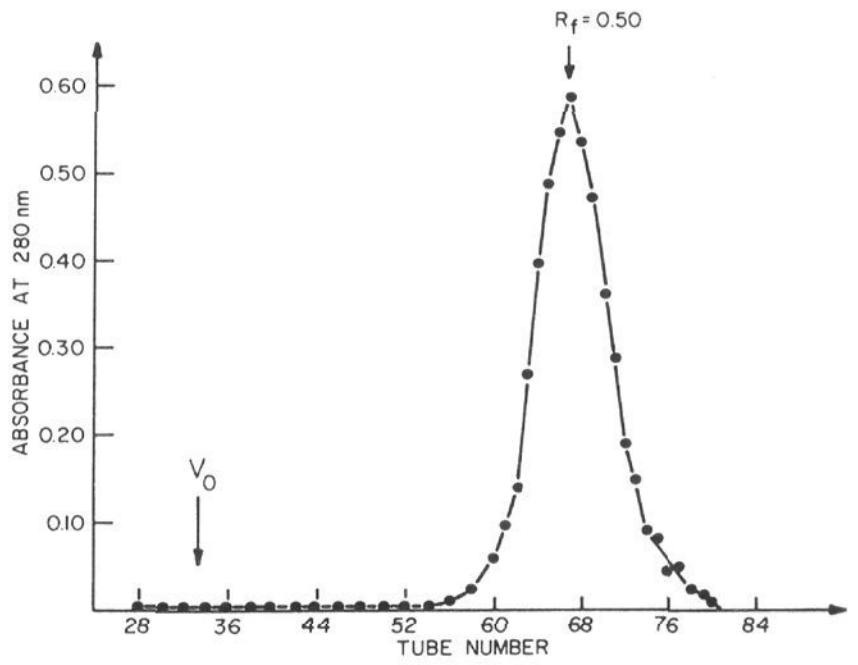

Figure 9. Gel filtration of highly purified synthetic cyclized somatostatin. Column: 115 × 0.6 cm Sephadex G-25F. V_0 = hold-up volume, 14.63 ml. Sample, 5 mg applied in 0.5 ml of 2 *N* AcOH.

direct inlet system. The cathode current was set at 1 mA with an ionizing potential of 70 eV. The accelerating voltage was maintained at 3 kV and the ion source temperature at 250°. The data were recorded by a Varian 620/i computer and a Varian Statos I plotter. The probe temperature for each analysis is listed underneath the respective spectrum.

Boc-Cys(*p*-OMe-Bzl)-polymer. Chloromethylation of the polymer (Bio-Beads SX-1, 200–400 mesh, from Bio-Rad Lab Inc.) was done according to Stewart and Young.[12] A substitution of 0.70 mequiv of Cl/g was obtained.

(a) To the chloromethylated polymer (10 g) suspended in absolute EtOH (50 ml) was added Boc-Cys(*p*-OMe-Bzl) (7 mmol, 2.38 g) and Et_3N (6.3 mmol, 0.98 ml). The suspension was refluxed for 36 hr. After thorough washes (MeOH, 12.5% Et_3N in DMF, DMF, MeOH, and CH_2Cl_2), the resin was dried under high vacuum; a weight gain of 1.15 g (0.34 mequiv/g) was consistent with a substitution of 0.32 mequiv/g obtained by Gisin test.[30]

(b) To the chloromethylated polymer (10 g) suspended in an-

(29) D. Yamashiro, *Nature* (*London*), **201**, 76 (1964).

(30) B. F. Gisin, *Anal. Chim. Acta*, **58**, 248 (1972).

hydrous DMSO (50 ml) was added Boc-Cys(*p*-OMe-Bzl) (7 mmol, 2.38 g) and $(CH_3)_3COK$ (6.3 mmol, 0.707 g). The suspension was warmed at 80° for 30 min. Washes included H_2O first, followed by the above-mentioned sequence. Weight gain and substitution obtained were within 10% of what was found under (a).

For determination of the optical purity of the esterified cysteine, a sample (1 g) of the above products was carried through standard solid phase synthesis procedures to prepare N^{α}-Boc-Leu-Cys(*p*-OMe-Bzl)-polymer. The dipeptide Leu-Cys-OH was obtained by concomitant deblocking and cleavage from the resin by HF (10 ml) in presence of anisole (1 ml). After elimination of HF and anisole under high vacuum, the resin was washed with 2 *N* AcOH and the filtrate immediately lyophilized. Iodoacetic acid (235 μmol, 44 mg) was added to the dried residues (214 μmol, 50 mg) dissolved in 21 ml of water. The pH was raised to 8 and the reaction completed in a few minutes. An aliquot (2 μmol, 200 μl) of both preparations was directly applied on a column for chromatography according to the procedure of Manning and Moore.[16] In this system, 2 nmol of the dipeptide L-Leu-D-Cys(Cm) (approximate retention time in our system, 333 min) would have been detected if present. L:Leu-L-Cys(Cm) (method a) (retention time, 310 min) was contaminated with 20% L-Leu (retention time, 500 min) and 4% of an unknown compound (retention time, 433 min). L-Leu-L-Cys(Cm) (method b) was contaminated with less than 1% of L-Leu and 2% of the same unknown compound (retention time, 433 min).

Protected Peptide-Resin of Somatostatin. N^{α}-Boc-Cys(*p*-OMe-Bzl)-resin (9 g) prepared as just described was placed in a 300-ml reaction vessel on a rocker for manual operation and was carried through the schedule shown in Tables I and II for the introduction of the remaining 13 amino acids. N^{α}-Boc protection was used for the following residues with the side-chain protecting groups indicated in parentheses: Ser(Bzl), Thr(Bzl), Phe, Trp, Lys(2-Cl-Z), Cys(*p*-OMe-Bzl). Alanine and glycine were introduced as the protected dipeptide Z-Ala-Gly-OH. The active ester program was used for the asparagine residue. Ser 13 was the only residue that consistently had to couple twice. The finished peptide-resin was dried under high vacuum to yield 12 g. An average 10 mg of resin/coupling was discarded for ninhydrin test. The amino acid analysis after HCl–propionic acid (1:1) hydrolysis for 2 hr at 130°[31] showed the following ratios: Asp, 1.0; 2Thr, 1.8; Ser, 0.6; Gly, 1.0; Ala, 1.2; 3Phe, 2.9; 2Lys, 1.8; NH_3, 1.1; Trp, 0.0; Cys, 0.0.

Reduced Somatostatin. Peptide-resin (12 g) was treated with HF (150 ml) for 0.75 hr at 0° in the presence of anisole (20 ml). After removal of HF and thorough drying under vacuum, the resin was washed with anhydrous ether (200 ml in portions), extracted with degassed 2 *N* AcOH (600 ml in portions) and immediately lyophilized. A white powder was obtained (1.8 g). This product (1.25 g) was dissolved in 2 *N* AcOH–10^{-2} M β-mercaptoethanol (12.5 ml) and was subjected to gel filtration on a 2.5 × 200 cm Sephadex G-25F column in the same solvent. Spectrophotometric measurements at 280 nm gave the profile indicated in Figure 1. Fractions of 670–740 ml yielded 500 mg (40% of applied amount) after lyophilization. This product was dissolved in 5 ml of the same solvent and reapplied on the same column. Two peaks were detected (280 nm), a small one at 500 ml and a large one at 600 ml. Isolation of the material represented by the larger peak gave 350 mg (28% of the original crude preparation) of what we call highly purified reduced somatostatin or linear tetradecapeptide. Several such preparations have been made at the writing of this paper, including some tenfold larger; results within conditions described are reproducible. Characterization of this peptide by amino acid analysis showed the following ratios after 6 *N* HCl hydrolysis containing 0.5% thioglycolic acid: Asp, 1.0; 2Thr, 2.0; Ser, 0.5; Gly, 1.0; Ala, 1.0; 3Phe, 2.8; 2Lys, 1.9; NH_3, 1.2; Trp, 0.5; 2Cys, 1.60 as half-cystine. After performic oxidation and the same conditions of hydrolysis: Ser, 0.9; Trp, 0.0, and 2Cys, 2.0 as cysteic acid. After enzymatic hydrolysis the same ratios were observed except for Trp, 1.0; 2Cys, 1.20 essentially as half-cystine and Asp as Asn. Under the conditions of electrophoresis described for the cyclized material (see Figure 6), we observed only one slightly tailing spot, uv and ninhydrin positive. Trypsin digestion for mass spectrometric analysis of the linear tetradecapeptide is identical with that described below for synthetic and cyclized somatostatin. Derivatization consisted of acetylation (acetic anhydride) followed by permethylation (sodium methylsulfinyl-methide and methyl iodide) according to Leclercq and Desiderio.[32] For a detailed procedure see Ling *et al.*[4]

Somatostatin. Reduced somatostatin (160 mg) was dissolved in 0.01 *M* NH_4OAc (1 l. in a 2-l. beaker). The pH was adjusted to 6.8. The solution was exposed in a cold room (8°) to air without stirring for 48–62 hr in the dark. Different concentrations of NH_4OAc from 1 *M* to plain water showed by approximate tlc analysis that best yields in cyclized material are obtained with concentrations of salt from 0.005 to 0.1 *M*, the concentration of somatostatin being constant (1 mg/10 ml). After two lyophilizations, most of the NH_4OAc has been eliminated and a white fluffy material is obtained. It partially dissolved in the upper phase of the BAW system (1.6 ml). After centrifugation, the solution is applied on a column (1.5 × 105 cm). The profile is shown in Figure 4. Tubes 92–106 were pooled to give 40 mg of pure somatostatin. CHNOS analysis shows the proper ratios for somatostatin · 3AcOH · $5H_2O$.

Anal. Calcd for $C_{82}H_{125}N_{18}O_{30}S_2$: C, 51.64; H, 6.61; N, 13.32; S, 3.36. Found: C, 51.61; H, 6.30; N, 13.24; S, 3.33.

Amino acid analyses under various hydrolysis conditions are shown in Table III along with the corresponding analyses of natural somatostatin. This material was found homogeneous by tlc in seven different solvent systems and behaved identically to the natural hormone in three of the systems (see Figure 5 for R_f values). It was also found homogeneous and identical with the natural somatostatin by electrophoresis under the conditions shown in Figure 6. Optical rotations were measured in 1% AcOH. Peptide concentrations were obtained on the basis of dry weight determination. Results obtained for natural and synthetic somatostatin are: $[\alpha]^{22}D$ −32.3 ±2° (*c* 0.244, 1% AcOH) and $[\alpha]^{22}D$ −33.3 ± 0.5° (*c* 1.0, 1% AcOH). These results are in full agreement when keeping in mind the presence of some nonpeptide contaminants in the natural hormone. Circular dichroism spectra of synthetic and natural somatostatin were measured in aqueous solutions after both compounds had been lyophilized from 0.1 *N* HCl solutions. The concentrations were 1.845 and 1.180 mg/ml, respectively, and were obtained subsequently by amino acid analysis for peptide content. The pH of the solutions was approximately 4. The measurements were done at 25° in a 0.1-mm cell. Slight discrepancies in the 230–250-nm region could be explained by the presence of some nonpeptide impurities in the solution of natural product.

Trypsin digestion of somatostatin (natural and synthetic) was performed according to Ling *et al.*[4] Somatostatin (50 μg, *ca.* 25 n*M*) was digested with 2 μg of trypsin in a 20-μl solution of 0.1 *M* NH_4OAc and 10^{-3} *M* $CaCl_2$ at pH 8.1 for 18 hr at 37°. After addition of 1 drop of 1 *N* AcOH the solution was lyophilized, taken in 1 drop of 50% AcOH, and analyzed on tlc (Figure 6). When subjected to gel filtration (Sephadex G-25F, column 0.6 × 115 cm), synthetic somatostatin (5 mg) shows a symmetrical elution pattern (Figure 9). The R_f of 0.50 obtained is identical with that of the natural somatostatin.[2]

Acknowledgments. We thank R. Burgus for the natural somatostatin used in our comparative studies, for measuring the specific optical rotation of the natural somatostatin, and for the amino acid analyses. Determination of the biological activity of natural and synthetic somatostatin was performed by W. Vale and P. Brazeau, whom we gratefully acknowledge. We are indebted to N. Ling for the elegant mass spectrometric studies. We thank M. Goodman for familiarizing us with his Cary 61 CD spectrophotometer. We appreciate the valuable technical help of M. Butcher, R. Kaiser, B. Lerch, R. Wolbers, and D. Neal. This research was supported in part by the Edna McConnell Clark Foundation grant to R. Guillemin and the National Institutes of Health Grant AM 16707-01 to W. Vale.

(31) J. Scotchler, R. Lozier, and A. Robinson, *J. Org. Chem.*, **35**, 3151 (1970). Ser, Cys, and Trp are unstable under those conditions.

(32) P. A. Leclercq and D. M. Desiderio, *Anal. Lett.*, **4**, 305 (1971).