SYNTHESIS OF A PEPTIDE WITH FULL SOMATOSTATIN ACTIVITY

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SUMMARY: A peptide has been synthesized according to the structure proposed for somatostatin by the solid phase method. The synthetic product was assayed and found to possess full somatostatin activity as compared with the natural material.

Inhibition of growth hormone (somatotropin) secretion from the pituitary gland by hypothalamic preparations has been previously reported by McCann and his co-workers (1). Recently, the isolation and structure of this somatotropin-release inhibiting factor from ovine hypothalamic tissue has been described by Guillemin et al. (2, 3). It is a tetradecapeptide, named somatostatin, with half-cystine at the COOH-terminus and contains a single tryptophan residue (see Figure 1). The structural features of somatostatin are of interest to us, especially from a synthetic point of view. We have recently used the solid phase method (4) to synthesize the COOH-terminal cyclic dodecapeptide (5) of the structure of human growth hormone (6) using 3,4-dimethylbenzyl protection of the sulfhydryl group of cysteine (5, 7). In addition, we have described the use of the formyl group for protection of the indole function of tryptophan in peptide synthesis (8). Both of these new protecting groups have been used in the synthesis of somatostatin as described herein.

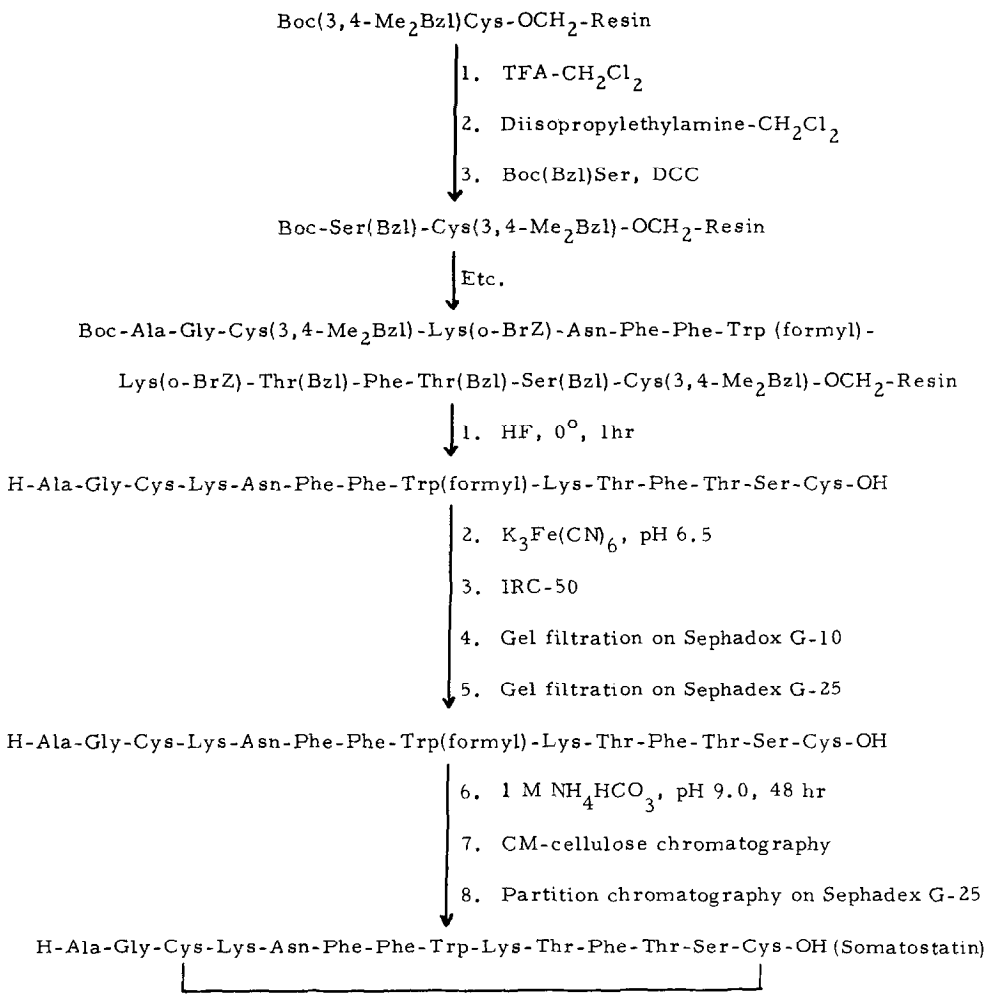

Figure 1. Outline of the synthesis of somatostatin by the solid phase method. Boc, t-butyloxycarbonyl; Bzl, benzyl; TFA, trifluroacetic acid; DCC, dicylcohexylcarbodiimide; o-BrZ, ortho-bromobenzyloxycarbonyl; CM-cellulose, carboxymethylcellulose.

The strategy for assembly of the linear peptide sequence by the solid-phase method is shown in Figure 1. Cleavage of the peptide from the solid support was performed with hydrogen fluoride (9) which removed all the protecting groups with the exception of the formyl group (10). Oxidative cyclization and deformylation were performed in two separate steps. Preliminary experiments showed that ring closure could be effected by air oxidation, but we chose to use the potassium ferricyanide

method (11) in order to avoid premature deformylation. During the course of oxidation considerable amounts of insoluble material were formed, even at a peptide concentration of 0.14 micromole per ml, indicating that ring closure may be accompanied by substantial formation of polymeric substances. The cyclized peptide, still containing the formyl group, was isolated as indicated in Figure 1. Essentially one peak was observed in the gel filtration on Sephadex G-25, and its position on the chromatogram indicated the product was a monomer. The product was treated with 1 M NH_4HCO_3 of pH 9 as previously described (8) to remove the formyl group from tryptophan. The material resulting from this treatment was then purified by chromatography on CM-cellulose (12) and by partition chromatography on Sephadex G-25 (13). The highly purified synthetic product was characterized by paper electrophoresis at two pH's, thin layer chromatography, amino acid analysis, exclusion chromatography on Sephadex G-25, and optical rotation. Its ultraviolet absorption spectrum was in agreement with that expected for somatostatin. The synthetic product has been assayed by the in vitro method (14) and found to possess 1.09 x the activity of the natural material with confidence limit of 0.52-2.39, and index of precision of 0.22 in a 4-points assay design.*

EXPERIMENTS AND RESULTS

Solid Phase Synthesis Procedures. Boc (3,4-dimethylbenzyl)-cysteinyl polymer was prepared from Boc (3,4-dimethylbenzyl)-cysteine (5,7) and chloromethylated resin by the Loffet method (15) according to a modified procedure recently described (16). Synthesis was started with

* The authors thank Drs. R. Guillemin and W. Vale for the assay of our synthetic product; detailed account of the assay data will be reported elsewhere.

2.62 g of this polymer with a load of 0.84 mmole of the protected cysteine derivative. The schedules employed for introduction of each of the remaining amino acid residues followed essentially those described for the synthesis of human ACTH (16) with the following exception: two equivalents were employed in all carbodiimide-mediated couplings which were used along with the following side-chain protecting groups: Lys, o-BrZ; Ser, Bzl; Thr, Bzl; Trp, formyl; Cys, 3,4-Me_2Bzl. The finished peptide resin was dried in vacuo over P_2O_5 to yield 4.61 g.

Peptide with the Proposed Somatostatin Structure. Protected peptide resin (2.03 g) was treated with HF (40 ml) for 1 hr at 0° in the presence of anisole (2 ml). After removal of HF and drying in vacuo, the resin was washed with ethyl acetate. The dried resin was extracted with trifluoroacetic acid (20 ml) and filtered off. The filtrate was evaporated in vacuo to an oil which was taken up in 25 ml of 50% aqueous acetonitrile. The sulfhydryl content was 440 micromoles as determined by the Ellman method (17). The solution was diluted with 1500 ml of water, the pH was adjusted to 6.5 with NH_4OH, and titrated with 0.01 M potassium ferricyanide until a permanent yellow color was observed. About 8 g Bio-Rad AG3-X4A resin (chloride form) was added to the very turbid solution, and the resin was filtered off after stirring. The filtrate was passed through a 3.5 x 4 cm column of the AG3-X4A resin in order to remove ferricyanide and ferrocyanide ions. The peptide material was absorbed on a 3 x 5 cm IRC-50 column and then eluted with pyridine-acetic acid-water (30:4:66, v/v). The eluate containing the peptide was concentrated to a small volume and place on a 2.2 x 25 cm Sephadex G-10 column. Elution with 0.5 M acetic acid and the collection of 3-ml

fractions gave a major peak as detected at 300 nm. Fractions 11-24 were combined, evaporated in vacuo to a low volume, and placed on a 2.5 x 136 cm Sephadex G-25 column. Elution with 0.5 M acetic acid gave a single sharp peak at an elution volume of 540 ml (column volume, 670 ml). Isolation by lyophilization gave 107.5 mg. For removal of the formyl group, a 30 mg sample was dissolved in 30 ml of 1 M NH_4HCO_3 of pH 9 and allowed to stand at room temperature for 48 hr by which time the absorption at 300 nm had declined to 18% of the initial value. The solution was lyophilized and the resulting product was subjected to chromatography on a 1.0 x 55 cm column of CM-cellulose initially equilibrated with 0.01 M ammonium acetate of pH 4.5. After elution with 105 ml of starting buffer, a gradient with respect to pH and salt concentration was effected by introducing 0.1 M ammonium acetate through a 500-ml mixing chamber containing the starting buffer. As detected spectrophotometrically at 280 nm two peaks were observed: a small one 40 ml after the buffer change, and a large one 390 ml after the change. The large peak gave 15.7 mg of peptide which was submitted to partition chromatography on Sephadex G-25 in a 1.9 x 23 cm column of Sephadex G-25 in the solvent system 1-butanol-0.2 M aqueous ammonium acetate (1:1, v/v) with collection of 1.4 ml fractions. The major peak emerged with R_f 0.42 and isolation of material corresponding to this peak gave 10.9 mg of highly purified synthetic product, $[\alpha]_D^{24}$ - 29° (c 0.5, 0.1 M acetic acid).

Paper electrophoresis (400 V, 3 hr, Whatman 3MM) in pyridine acetate buffer (pH 3.7) and in collidine acetate buffer (pH 6.9) gave single ninhydrin positive spots with R_f values of 0.50 and 0.44 respectively, relative to lysine. Thin layer chromatogrophy in 1-

butanol-pyridine acetic acid-water (30:20:6:24, v/v) gave a single ninhydrin positive spot with R_f 0.47. Amino acid analysis of a toluenesulfonic acid hydrolysate (18) gave $Trp_{0.9}$ $Lys_{2.2}$ $Asp_{1.0}$ $Thr_{1.8}$ $Ser_{1.0}$ $Gly_{1.0}$ $Ala_{1.1}$ $Phe_{3.0}$ and half-cystine$_{1.8}$. The ultraviolet spectrum of the peptide taken in the range 240-360 nm showed the characteristics of tryptophan. Gel filtration on a 1.3 x 112 cm Sephadex G-25 column (column volume, 166 ml) gave a single peak with elution maximum at 133 ml.

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REFERENCES

1. Krulich, L, Dharrival, A. P. S., and McCann, S. M., Endocrinology 83, 783 (1968).
2. Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., and Guillemin, R., Science 179, 77 (1973).
3. Burgus, R., Ling, N., Butcher, M., and Guillemin, R., Proc. Nat. Acad. Sci. USA 70, 684 (1973).
4. Merrifield, R. B., Biochemistry 3, 1385 (1964).
5. Yamashiro, D., Noble, R. L., and Li, C. H., J. Org. Chem., in press.
6. Li, C. H., Dixon, J. S., and Liu, W. -K., Arch. Biochem. Biophys. 133, 70 (1969); Li, C. H., and Dixon, J. S., Arch. Biochem. Biophys. 146, 233 (1971); Li, C. H., Proc. Amer. Phil. Soc. 116, 365 (1972).
7. Yamashiro, D., Noble, R. L., and Li, C. H., in "Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium," J. Meienhofer, ed., Ann Arbor Science Publishers, Ann Arbor, 1972, p. 197.
8. Yamashiro, D., and Li, C. H., J. Org. Chem., in press.
9. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H., Bull Chem Soc. Japan 40, 2164 (1967); Lenard, J., and Robinson, A. B., J. Amer. Chem. Soc. 89, 181 (1967).
10. Ohno, M., Tsukamoto, S., Makisumi, S., and Izumiya, N., Bull. Chem. Soc. Japan 45, 2852 (1972).

11. Hope, E. B., Murti, V. V. S., and du Vigneaud, V., J. Biol. Chem. 237, 1563 (1962).
12. Peterson, E. A., and Sober, H. A., J. Amer. Chem. Soc. 78, 751, (1956).
13. Yamashiro, D., Nature (London) 201, 76 (1964).
14. Vale, W., Grant, G., Amoss, M., Blackwell, R., and Guillemin, R., Endocrinology 91, 562 (1972).
15. Loffet, A., Int. J. Protein Res. 3, 297 (1971).
16. Yamashiro, D., and Li, C. H., J. Amer. Chem. Soc. 95, 1310 (1973).
17. Ellman, G. L., Arch. Biochem. Biophys. 82, 70 (1959).
18. Liu, T. Y., and Chang, Y. H., J. Biol. Chem. 246, 2842 (1971).