THE USE OF MASS SPECTROMETRY IN DEDUCING THE SEQUENCE OF SOMATOSTATIN - A HYPOTHALAMIC POLYPEPTIDE THAT INHIBITS THE SECRETION OF GROWTH HORMONE

N. Ling, R. Burgus, J. Rivier, W. Vale and P. Brazeau

The Salk Institute, La Jolla, California 92037

Received November 14,1972

SUMMARY: A peptide has been isolated from the extracts of ovine hypothalamic tissues that inhibits the secretion of somatotropin (growth hormone). The primary structure of this peptide, named somatostatin, has been determined to be H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH. The sequence -Asn-Phe-Phe-Trp-Lys- was confirmed by direct mass spectrometry, using an acetylated and permethylated tryptic digest of somatostatin.

We recently reported the isolation of a polypeptide from ovine hypothalamic extracts which inhibits the release of growth hormone *in vitro* and *in vivo* (1). This peptide, to which we have given the name somatostatin, was found to contain the following amino acids (2): Ala 1, Gly 1, Thr 2, Lys 2, Phe 3, Ser 1, Cys 2, Trp 1, Asn 1. Direct sequencing of somatostatin was attempted by stepwise Edman degradation performed on the carboxymethylated peptide (2). However, after cleavage of the first five amino acids, H-Ala-Gly-Cys-Lys-Asn, the remaining phenylthiocarbamyl-protected peptide partitioned into the organic phase which rendered the interpretation of the ensuing Edman degradation difficult.

Since somatostatin has two lysines and we already knew the position of one of them, we decided to cleave the peptide with trypsin into two or three smaller peptides, depending on the position of the remaining lysine (2). The unresolved tryptic digest was subjected to Edman degradation. After the first Edman cycle, we found two peptides in the aqueous phase. Since we already knew that the sequence of one of them was H-Ala-Gly-Cys-Lys-OH, the sequence of the other peptide was deduced as H-Thr-Phe-Thr-Ser-Cys-OH by the Edman procedure. These results

Copyright © 1973 by Academic Press, Inc.
All rights of reproduction in any form reserved.

implied that there was a third peptide containing the following amino acids: Asn 1, Phe 2, Trp 1, Lys 1, which was soluble in the organic phase after the phenylisothiocyanate treatment. Since this peptide was within the scope of direct sequencing by mass spectrometry, we prepared a suitable derivative of this compound for analysis by acetylation and permethylation (3).

MATERIAL AND METHODS

Trypsin Digestion of Somatostatin: Somatostatin (450 nM) was digested with 40 µg of trypsin (Worthington) in a 200 µl solution of 0.1M NH_4OAc and 10^{-3}M $CaCl_2$ at pH 8.1 for 15½ hr at 37°. An aliquot corresponding to 270 nM of somatostatin was withdrawn and lyophilized. To the lyophilized residue, 50 µl of 0.1M HOAc was added and the solution again lyophilized. The sample was then further dried overnight in vacuum at 80° to remove the excess NH_3.

Acetylation and Permethylation: The dried sample was taken up in 100 µl H_2O and 3.5 µl (37 µM) of redistilled $(CH_3CO)_2O$ was added, followed by 5 mg (61 µM) of NaOAc in 12.5 µl H_2O. The mixture was agitated in the cold room for 1 hr and then evaporated to dryness. The excess acetate ion was removed by addition of 100 µl of CH_3OH saturated with HCl and evaporation to dryness. The residue was taken up in 100 µl H_2O and the acetylated peptide extracted with 4X100 µl $CHCl_3$. The $CHCl_3$ phase was evaporated and dried for 1 hr in vacuum at 80°.

A 0.5 molar solution of $NaCH_2SOCH_3$ was prepared in the following manner: 200 mg NaH (50% oil suspension, Gallard-Schlesinger Chem. Corp.) was washed with 5X2 ml anhyd Et_2O and then suspended in 8 ml DMSO. The suspension was kept under argon and heated at 65° in an oil bath until hydrogen ceased to evolve (~1 hr).

One hundred microliters (50 µM) of the clear sodium methylsulfinylmethide solution were added to the residue from the $CHCl_3$ extract, agitated for 5 min, followed by 6.2 µl (100 µM) of CH_3I and the mixture

agitated for 1 hr. The reaction was terminated by the addition of 3 drops of H_2O and the solution evaporated to dryness in vacuum at 80°. The resulting residue was taken up in 100 µl H_2O and extracted with 3X100 µl $CHCl_3$. After concentration, the residue from the $CHCl_3$ phase was transferred into a gold crucible for mass spectrometric analysis.

Mass Spectrometry: All samples were run in a Varian Mat CH-5 mass spectrometer with the 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.

RESULTS AND DISCUSSION

In general, the mass spectra of N-acetylated and permethylated peptides exhibit peaks which correspond to the cleavage of the CO-N bond with charge retention on the carbonyl portion of the molecule (4). In higher oligopeptides, these peaks are often the most intense ones and seem to occur by a sequential splitting of one amino acid residue after the other, starting from the molecular ion. As a result, the sequence of an unknown peptide can be deduced if the various acylium ions are identified. These acylium ions, in turn, may or may not lose carbon monoxide to give peaks that are 28 mass units lower.

For our present case, the peptide extracted from a tryptic digest of somatostatin gave a very clean mass spectrum after derivatization (see Fig. 1). Since we knew that the peptide contained 1 Asn as the N-terminus, 1 Lys as the C-terminus, 2 Phe and 1 Trp, it was not difficult to deduce the complete sequence as H-Asn-Phe-Phe-Trp-Lys-OH from the acylium ions at m/e 199, 360, 521, 735, 933 in the mass spectrum. Even the molecular ion of the derivatized peptide was observable at m/e 964. That Trp was coupled to Lys was confirmed by the ion at m/e 413 which

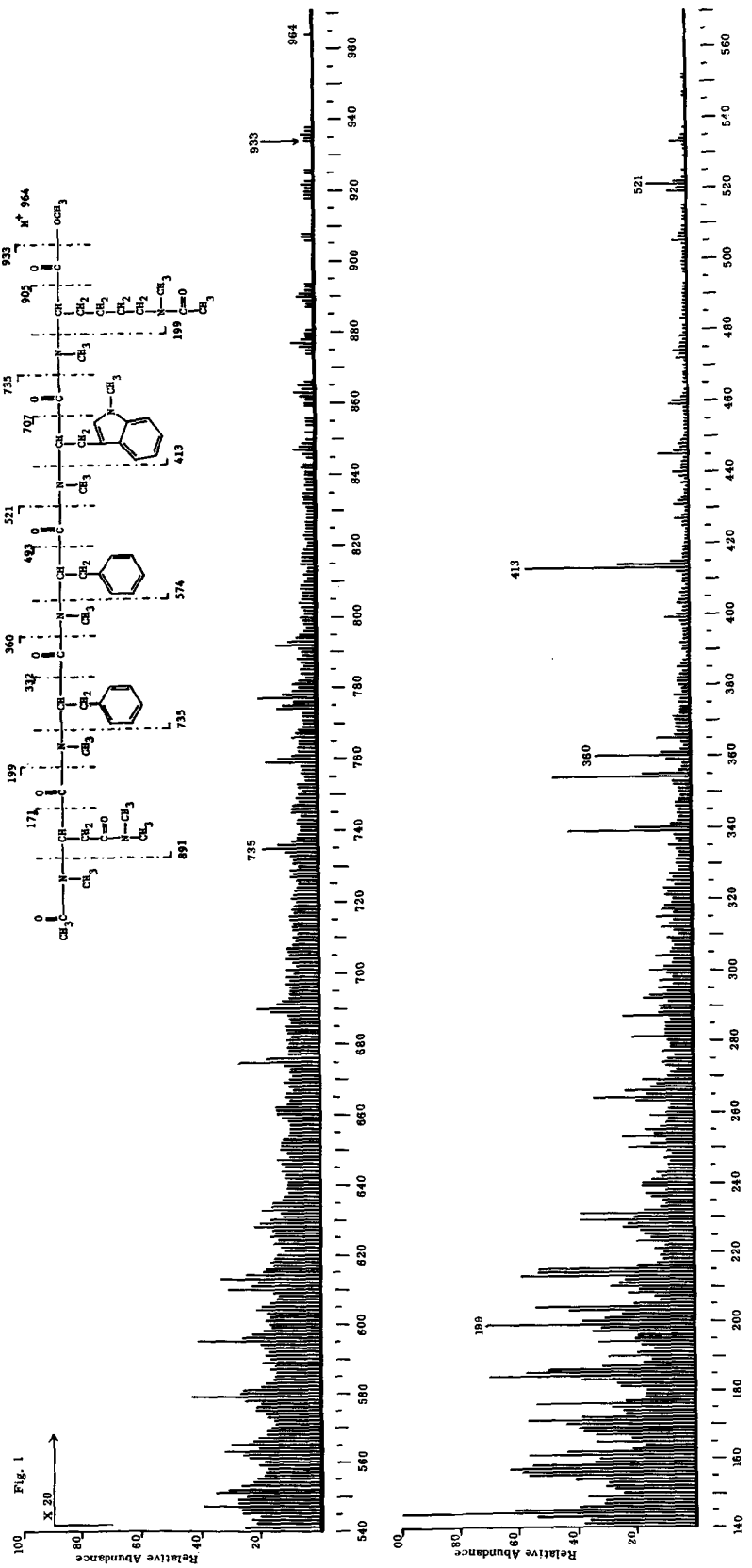
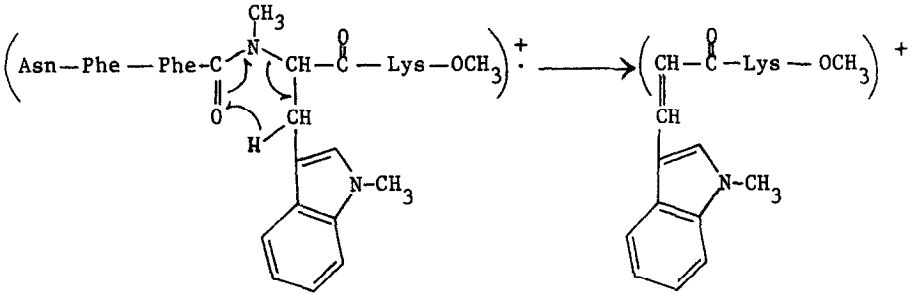


Fig. 1. Mass spectrum of a permethylated chloroform extract from an acetylated tryptic digest of natural somatostatin. Probe temp. = 285°.

resulted from cleavage of the N-C bond with a concurrent hydrogen transfer to the oxygen and charge retention on the indole portion of the molecule. This type of rearrangement ions, namely: m/e 199, 574, 735, 891, were not observed for the Lys, Phe, Asn residues because they did not possess a charge stabilizing heterocyclic ring.



m/e 964 m/e 413

Having deduced the sequence of this peptide, we could readily fit it with the other two peptides derived from the trypsin digestion, namely: H-Ala-Gly-Cys-Lys-OH and H-Thr-Phe-Thr-Ser-Cys-OH, and arrived at the sequence of somatostatin as H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH*. When a synthetic replicate of the linear peptide (5) was subjected to trypsin digestion and the same derivatization process, the mass spectrum obtained (see Fig. 2) was practically identical to the one from the natural somatostatin. When CH_3I was substituted with CD_3I for the permethylation, the sequence containing peaks all shifted to the predicted values, indicating that the assignment for the various acylium ions was correct.

* Somatostatin, as isolated, contains a disulfide bridge between the two cysteines as shown by studies with 5,5'-dithiobis-(2-nitrobenzoic acid)(2).

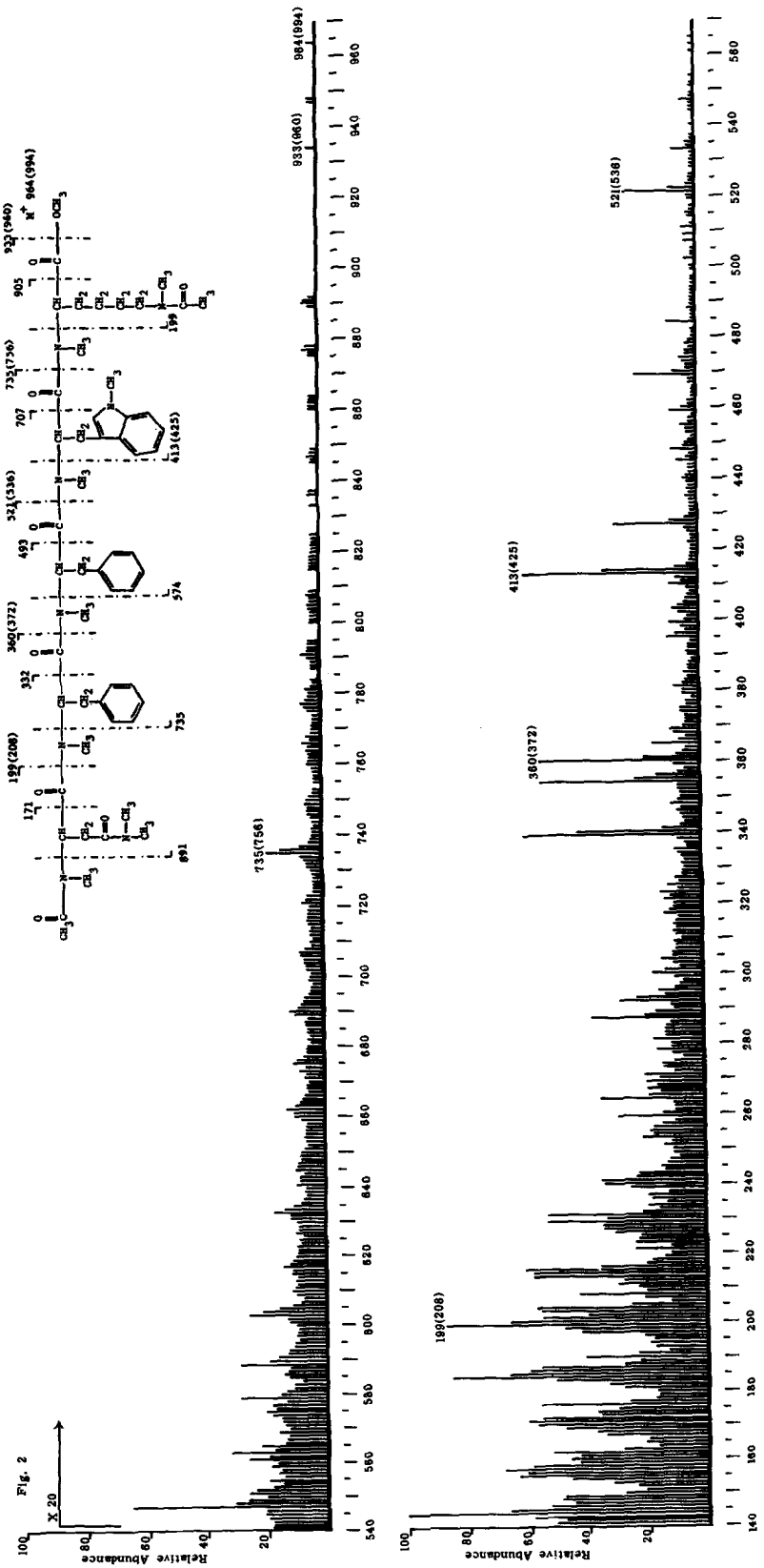


Fig. 2. Mass spectrum of a permethylated chloroform extract from an acetylated tryptic digest of synthetic somatostatin. Probe temp. = 281°. ( ) indicate peak shift when CH_3I was substituted with CD_3I in the permethylation.

ACKNOWLEDGEMENTS

We are grateful to the excellent technical assistance offered by D. Erenea, C. Garcia, R. Kaiser, R. Wolbers and M. Butcher. We also thank B. Dorscht for typing this communication.

This research is supported by AID (Contract No. AID/csd 2785 to Dr. R. Guillemin), Ford Foundation and Rockefeller Foundation.

REFERENCES

1. A preliminary communication on the structure and biological activity of somatostatin has been published. See P. Brazeau et al., Science, (accepted for publication).

2. The methodology involved will be reported in detail by R. Burgus et al. (paper in preparation).

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

4. Das, B.C. and E. Lederer, In Topics in Organic Mass Spectrometry, A.L. Burlingame (ed.) Wiley-Interscience, New York, 1970, p. 286.

5. The synthesis of somatostatin will be reported in detail by J. Rivier et al., (paper in preparation).