MASS SPECTROMETRY OF *N*-PERMETHYLATED PEPTIDE DERIVATIVES; ARTIFACTS PRODUCED BY *C*-METHYLATION*

David W. THOMAS
Institut de Chimie des Substances Naturelles, C.N.R.S., 91-Gif-Sur-Yvette, France

Received 7 August 1969

1. Introduction

For peptide sequence determination by mass spectrometry, the value of *N*-permethylated peptide derivatives has been well established (for a review, see ref. [2]), and a number of methods have been developed to accomplish *N*-permethylation. The original Kuhn method, employing methyl iodide and silver oxide [3–5], has now been generally replaced by the Hakomori method [6–8], which makes use of methyl iodide and a methyl-sulfinyl carbanion in dimethylsulfoxide. The latter method was found to be successfully applicable to a larger variety of amino acid residues than was the Kuhn method. Recently, a technique similar to the Hakomori method has been suggested [9] which uses dimethylacetamide as solvent and a base generated from dimethylacetamide and sodium hydride.

A method previously known for *O*-permethylation of carbohydrates [10] has been proposed by Coggins and Benoiton [11] as effective for *N*-permethylation of peptides. This method appeared to offer a practical advantage over the former techniques: a simple heterogeneous mixture of methyl iodide, sodium hydride, and the peptide derivative in dimethylformamide precludes the prior preparation of a reagent. For this reason, the Coggins-Benoiton procedure was adopted in our laboratory as the preferable method for *N*-permethylation, and was found to be successful for most of our problems. For example, the largest peptides yet examined by mass spectrometry, of 18 and 22 amino acid residues, were analyzed after permethylation by this procedure [1,12].

However, with these reaction conditions, we encountered *C*-methylation of some amino acid residues under certain circumstances, particularly when working with sub-milligram peptide quantities. Some examples here will demonstrate the drawbacks of permethylation according to the method of Coggins and Benoiton [11]. In the following discussion, the advantages and disadvantages of the various methods proposed for permethylation of peptides will be evaluated.

2. Experimental

For permethylation by the Coggins-Benoiton technique, the peptide derivative (1–10 mg) is dissolved in 0.2 ml of dimethylformamide. Sodium hydride oil suspension (50 mg) is rinsed three times with ether, then added to the peptide solution, followed by 0.1 ml of methyl iodide. After one-half to one hr, at room temperature with stirring, the mixture is added to water and the product extracted with chloroform.

Conditions for permethylation of peptides by the Hakomori method have previously been described [8].

Mass spectra are determined with an A.E.I. model MS 9 mass spectrometer.

* Part XIX in the series "Determination of amino acid sequences in oligopeptides by mass spectrometry"; part XVIII, ref. [1].

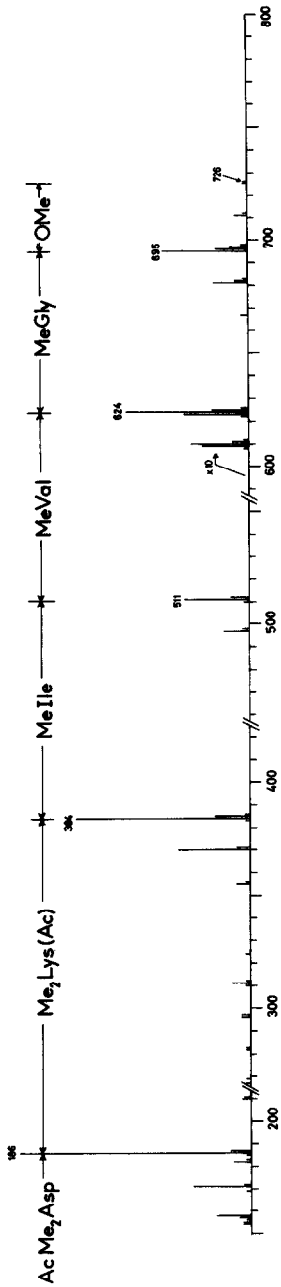

Fig. 1. Mass spectrum of product obtained after permethylation of Ac.Asp.Lys(Ac).Ile.Val.Gly.OH by the Hakomori method. Scale *m/e*.

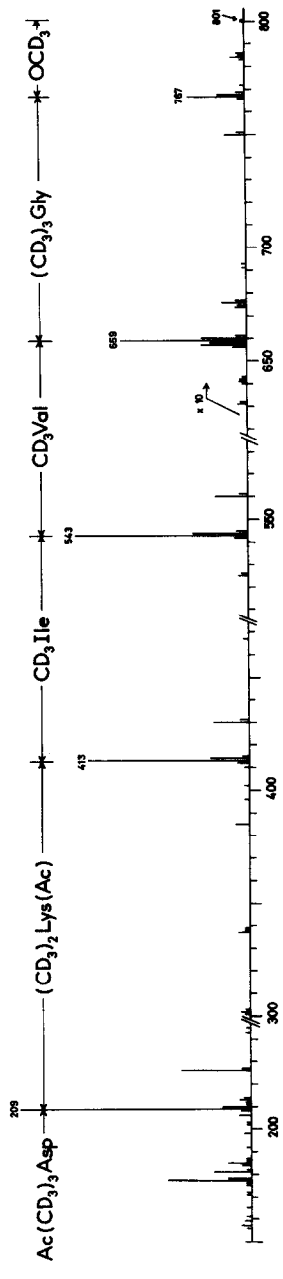

Fig. 2. Mass spectrum of product obtained after perdeuteriomethylation of Ac.Asp.Lys(Ac).Ile.Val.Gly.OH by the Coggins and Benoiton method. Scale *m/e*.

3. Results

The aspartic acid residue is the most susceptible to the side reaction of *C*-methylation, an example of which is illustrated with compound (1). Permethylation by the Hakomori method gave the expected *O,N*-permethyl derivative (2), the mass spectrum of which is shown in fig. 1. The structure was confirmed by this spectrum, and no abnormalities were observed except the reluctance toward methylation of the ε-nitrogen of lysine (see *m/e* 370) which has previously been noted [5].

Ac.Asp.Lys(Ac).Ile.Val.Gly.OH (1)

$Ac.Me_2Asp.Me_2Lys(Ac).MeIle.MeVal.MeGly.OMe$ (2)

By comparison, use of the Coggins-Benoiton conditions as described above (using deuteriomethyl iodide) produced a derivative which gave the mass spectrum of fig. 2. A significant observation is the lack of the expected fragment $Ac.NCD_3\underset{\underset{OCD_3}{|}}{Asp}.^+$ at *m/e* 192. Instead, the predominant product contained one *additional* CD_3 group (*m/e* 209), and was even accompanied by a smaller amount of a higher homolog (*m/e* 226). *C*-methylation of aspartic acid residues has also been observed in the following peptide derivatives†:

RCO.Glu.Leu.Leu.Val.Asp.Leu.Leu.OH (3)

Ac.Val.Ala.Val.Ile.Asp.Ser.Gly.(Ile.Asp.-Ser.Ser.His.Pro.Asp.Leu.Lys.OH) (4)

Ac.Asp.Orn(Ac).Val.Tyr.Ile.(Tha*.Pro.Phe.OH) (5)

One and two additional *C*-methyl groups were also found in the glycine residue of the methylation product of compound (1) (see fig. 2). A similar *C*-methylation of glycine, to give alanine or a higher homolog, has been observed for the insulin derivative (6) and the bradykinin derivative (7). *C*-methylation of glycine residues has also been obtained with the Kuhn method [9], although this method has already been generally discontinued for peptides for other reasons [8,13].

Ac.Gly.Ile.Val.Glu.GlN.(Cys(SO_3H). . . .OH) (6)

Ac.Orn(Ac).Pro.Pro.Gly.Phe.Ser.(Pro.Phe.Orn-(Ac).OH) (7)

The glutamic acid residue of compound (3) (and also of (6)) had a tendency to incorporate one additional methyl group, but to a lesser extent than the aspartic acid residue of the same peptide derivative.

4. Discussion

Although the Coggins-Benoiton reaction [11] is a very simple and effective method for peptide *N*-permethylation, the results presented here indicate that caution must be taken in the interpretation of mass spectra of the resulting derivatives, particularly when they contain aspartic acid, glutamic acid, or glycine. For example, *C*-methylation of an aspartic acid residue gives a homolog which may be erroneously identified by its mass as an *O,N*-dimethylglutamic acid residue. Likewise, glycine may be converted to *N*-methylalanine; the resulting mass spectrum is no longer representative of the original peptide.

The products of the side reaction pointed out above have not yet been characterized: it was considered that an additional methyl group could be introduced by *O*-methylation of an enol, e.g. $\ldots NME\text{-}\underset{\underset{R}{|}}{CH}\text{-}CO\ldots \rightarrow \ldots NMe\text{-}\underset{\underset{R}{|}}{C}{=}\underset{\underset{OMe}{|}}{C}\ldots$, but this cannot account for the *two* methyl groups (in addition to *N*-methyl) occasionally introduced in glycine residues; thus *C*-methylation is the only remaining possibility.

The products resulting from such *C*-methylation may be recognized and distinguished from naturally occurring homologs by use of deuteriomethyliodide, as was done for the example of fig. 2; but if a peptide is known to contain one of the problematic residues, especially the more reactive aspartic acid residue, it seems best to choose an alternative method of permethylation.

The reaction of Kuhn [3], which is successful for the *N*-permethylation of peptides [4], utilizes silver

† Residues in parentheses were not observed in mass spectra of the permethylated derivatives.
* Thienylalanine.

oxide and methyl iodide in dimethylformamide. This reaction was originally inconvenient because of its three-day time requirement, but this was later shortened to three hours by maintaining the reaction mixture at 50° in a sealed tube [5]. However, artifacts which limited the general usefulness of this method were produced with peptides containing aspartic acid, glutamic acid, methionine, and tryptophan [8,13].

The methylation method of Hakomori [6,7] has been modified [8] to produce clean *N*-permethylated peptide derivatives after 1 hr reaction with methyl iodide and a methylsulfinyl carbanion in dimethylsulfoxide. This procedure was found to be successful for the largest variety of peptides; none of the artifacts produced by the Kuhn or Coggins and Benoiton methods have been encountered. Thus it seems that the Hakomori method is still the most generally useful. The only inconvenience of this method (which may be avoided by use of the Coggins and Benoiton method, where applicable) is the additional step of preparing a carbanion catalyst by reaction of sodium hydride and dimethylsulfoxide at elevated temperature.

The significant difference between all methylation techniques is not in the solvent, but rather the base which is employed. In the technique of Coggins and Benoiton, the base is sodium hydride in suspension. Although dimethylformamide has usually been used as solvent, this reaction may be accomplished in dimethylsulfoxide, whereby *C*-methylation is also observed. The Hakomori method utilizes sodium hydride for the preparation of a reagent, the resulting base being a methylsulfinyl carbanion and not sodium hydride itself. It is the difference in base which must account for the degree of reactivity of the various methods.

Another technique for permethylation of peptides uses dimethylacetamide in place of the dimethylsulfoxide of the Hakomori reaction [9]. It has been suggested that this technique (which uses a reaction time of 1 hr) gives a cleaner peptide derivative than that obtained by the Hakomori reaction, although this may be due to a difference in reaction times (ref. [9] quotes only the original application of the Hakomori method to peptides, which involves a reaction time of 12 hr [7]; recent work has shown that prolongation beyond one-half to 1 hr leads to decomposition of the peptide derivative). This reaction, however, is still a "Hakomori" reaction in the sense that a base is prepared by prior reaction of sodium hydride with the solvent; and as with the Hakomori technique, no undesired artifacts have been reported.

Acknowledgements

The author wishes to thank Professor E.Lederer for his interest in this work, and Drs. R.Boissonnas and St.Guttmann (Sandoz, S.A.), F.M.Bumpus (Cleveland), J.Savrda (Orsay), and E.L.Smith (UCLA) for the compounds used in this research.

References

[1] G.H.deHaas, F.Franěk, B.Keil, D.W.Thomas and E.Lederer, FEBS Letters 4 (1969) 25.
[2] E.Lederer, 5th IUPAC Symposium on the Chemistry of Natural Compounds, London, July 1968. Pure Appl. Chem. 17 (1968) 489.
[3] R.Kuhn, I.Low and H.Trischmann, Ber. 90 (1957) 203.
[4] B.C.Das, S.D.Gero and E.Lederer, Biochem. Biophys. Res. Commun. 29 (1967) 211.
[5] D.W.Thomas, B.C.Das, S.D.Gero and E.Lederer, Biochem. Biophys. Res. Commun. 32 (1968) 199.
[6] S.I.Hakomori, J. Biochem. 55 (1964) 205.
[7] E.Vilkas and E.Lederer, Tetrahedron Letters (1968) 3089.
[8] D.W.Thomas, Biochem. Biophys. Res. Commun. 33 (1968) 483.
[9] K.L.Agarwal, G.W.Kenner and R.C.Sheppard, J. Am. Chem. Soc. 91 (1969) 3096.
[10] J.S.Brimacombe, B.D.Jones, M.Stacey and J.J.Willard, Carbohydrate Res. 2 (1966) 167.
[11] J.Coggins and L.Benoiton, Abstracts of Papers, Biol. 18 156th ACS National Meeting, Atlantic City, September 1968.
[12] F.Franěk, B.Keil, D.W.Thomas and E.Lederer, FEBS Letters 2 (1969) 309.
[13] K.L.Agarwal, R.A.W.Johnstone, G.W.Kenner, D.S.Millington and R.C.Sheppard, Nature 219 (1968) 498.