MASS SPECTRAL IDENTIFICATION AND QUANTIFICATION OF PHENYLTHIOHYDANTOIN DERIVATIVES FROM EDMAN DEGRADATION OF PROTEINS: CYSTEINE DERIVATIVES

Harald TSCHESCHE, Michael SCHNEIDER and Elmar WACHTER
Organisch-Chemisches Laboratorium der Technischen Universität München, Lehrstuhl für Organische Chemie und Biochemie, D-8 München 2, W. Germany

Received 27 March 1972
Original figures received 18 May 1972

1. Introduction

In the elucidation of amino acid sequences of proteins and peptides using the stepwise and sequential degradation of Edman [1] an unequivocal qualitative identification and a sensitive quantification is desirable. This has become an even more important demand since automated methods for sequencing have become available [2].

Mass spectral identification of the amino acid phenylthiohydantoin (PTH) derivatives formed during Edman degradation of peptides and proteins has been in use as a powerful tool for analysis during the last few years in our laboratory [3–9]. Quantification of this method has been achieved by using deuterated PTH derivatives as internal standards [8]. Especially the *p*-bromo-substituted phenylthiohydantoins characterized by a significant double peak of a mass difference of 2 amu for the molecular ion peak facilitated identification and interpretation of the relevant mass spectra. Mass spectrometry is superior to other methods of analysis based on thin-layer chromatography [10, 11] or gas–liquid chromatography [12, 13], since identification is directly correlated to individual mass numbers of the phenylthiohydantoin under investigation. Electron impact mass spectrometry, however, suffered from the difficulty of cysteine identification. The intensity of the molecular ion peak of several common derivatives is quite low or undetectable and the predominant fragment ion overlaps with the one formed from the PTH of serine.

We wish to report on an investigation of several PTH derivatives of cysteine and their mass spectral analysis and quantification. The *S*-methyl-cysteine derivative is by far the most suitable derivative for identification, since it may be formed conveniently under mild conditions in high yield, exhibits a stability and volatility comparable to methionine and generates the molecular ion as the predominant peak with a relative abundance of 100%.

2. Materials and methods

Cysteic acid was purchased from Serva GmbH, Heidelberg, and cysteine (Cys) from Fluka AG, Buchs. All reactions concerned with derivatization of Cys were carried out under a barrier of nitrogen. Prior to reaction Cys was reduced with a 3–5 molar excess of dithiothreitol at pH 8.0 in 50% pyridine/water for 60 min.

S-Carboxymethyl-Cys (CMCys) was prepared as already described [14].

S-Aminoethyl-Cys (AECys) was synthesized by reacting Cys with ethylenimine according to [15, 16], but performing the reaction in 50% pyridine/water for 30 min at room temp. after dithiothreitol reduction. *S*-Vinylpyridyl-Cys (VPCys) was obtained from reaction of Cys with a 5 molar excess of fresh distilled vinylpyridine for 120 min in 50% pyridine/water [17]. Excess reagent and solvent were removed by rotary evaporation and the residue was desalted on Bio Gel P-2, equilibrated with 0.1 M acetic acid.

S-Methyl-Cys (MeCys) could be conveniently prepared by addition of methyl ester of *p*-nitrobenzenesulfonate [18] to a solution of Cys in 50% pyridine/water at 40°, 30 min reaction time. The mixture was evaporated to dryness and the derivative purified by gel filtration on Bio Gel P-2, equilibrated with 0.1 M acetic acid.

p-Bromosubstituted phenylisothiocyanate and its tetradeuterated analogue were prepared from aniline or pentadeuteroaniline, respectively. The following route of synthesis was performed (given for the pentadeutero-derivative, 1–5):

1. Pentadeutero-aniline (15 ml), dissolved in 60 ml benzene, was slowly added under stirring to 15.5 ml acetic anhydride. The mixture was kept overnight in the refrigerator and the precipitated acetanilide filtered off. The mother liquor was concentrated by evaporation and washed with Na_2CO_3-solution. Additional acetanilide, which precipitated on cooling, was collected. The yield was 90% d_5-*N*-acetylaniline [18] (96% d_5). Fp. 116–117°.
2. Bromination was carried out by slow addition (1–1.5 ml/min) of 23 g Br_2 in 70 ml glacial acetic acid to a stirred solution of 20.1 g d_5-aniline in 140 ml glacial acetic acid, to which 12 g sodium acetate had been added. The reaction product was precipitated by pouring the mixture into 800 ml of aqueous sodium acetate, which was neutralized by addition of solid Na_2CO_3. The precipitate formed overnight was filtered. The yield was 95% *p*-bromo-d_4-*N*-acetylaniline [19]. Fp. 168–169°.
3. The protecting *N*-acetyl group was cleaved by acid hydrolysis for 105 min in 100 ml DCl (20% DCl in D_2O). The mixture was cooled and the precipitate was filtered. The mother liquor was concentrated by rotary evaporation and the residual precipitate added to the first. The hydrochloride was treated with a solution of Na_2CO_3 in order to obtain the free base. The yield was about 100% *p*-bromo-d_6-aniline [20]. Fp. 64–65°.
4. Conversion of the *p*-bromo-d_6-aniline into *p*-bromo-d_4-phenylisothiocyanate was performed using the procedure of Van der Kerk et al. [21]. Fp. 57–58° (94.7% d_4, ~ 5% d_3).

The phenylthiohydantoin (PTH) derivatives were obtained by reacting 2 mM Cys derivative in a pH-Stat (Autotitrator Radiometer, Copenhagen) with 1.5 molar excess of *p*-bromo-phenylisothiocyanate in 20 ml 50% pyridine/water titrated to pH 9.0 with 0.1 N NaOH at 25°. When the reaction had come to an end (end of NaOH consumed) the mixture was extracted 3 times with benzene or hexane/ethylacetate 5 : 1 (v, v) and evaporated to dryness. The residue was dissolved in 10 ml water and the solution acidified under stirring by dropwise addition of 1 N HCl. The solution was stirred at room temp. for 24 hr and the precipitate formed was collected.

All mass spectra were recorded on a MS 9 mass spectrometer (AEI, Manchester) equipped with the standard solid probe inlet system. Ionizing voltage was 12 eV (or 20 eV). Ion source temperature 190–220°.

3. Results and discussion

The mass spectra of six derivatives of cysteine are presented in figs. 1–6 (table). Cysteic acid gives the most stable PTH derivative, but is less well suited for mass spectrometry due to its most polar character. This polarity as well prevents convenient extraction with organic solvents from the reaction mixture after Edman degradation of the oxidized polypeptide chain. A molecular ion peak could not be obtained in detectable amounts in the mass spectrum, fig. 1. The fragment 282/84 amu $(M-SO_3H)^+$ representing the heterocyclic ring without the characteristic cysteic acid side chain was the only fragment formed in the upper mass range. The same fragment is obtained from all cysteine derivatives and is as well formed from serine *p*BrPTH and is therefore not characteristic for cysteic acid.

S-Carboxymethylcysteine pBrPTH produces the same fragment 282/84 amu, but in addition gives rise to a fragment 354/56 (about 3% rel. abundance) by elimination of a molecule of water and of hydrogen. Probably this ion is derived from a stabilized fragment formed via formation of a second condensed ring from the carbonyl of the carboxymethyl side chain with the α-amino nitrogen. Confirmation of the identity of the CMCys-*p*BrPTH may be obtained by identification of the fragment at 92 amu, which is predominating in the low mass range and is caused by fragmentation of the side chain producing thiolacetic acid ($HSCH_2COOH$). The molecular ion could not be obtained using electron impact mass spectrometry, fig. 2.

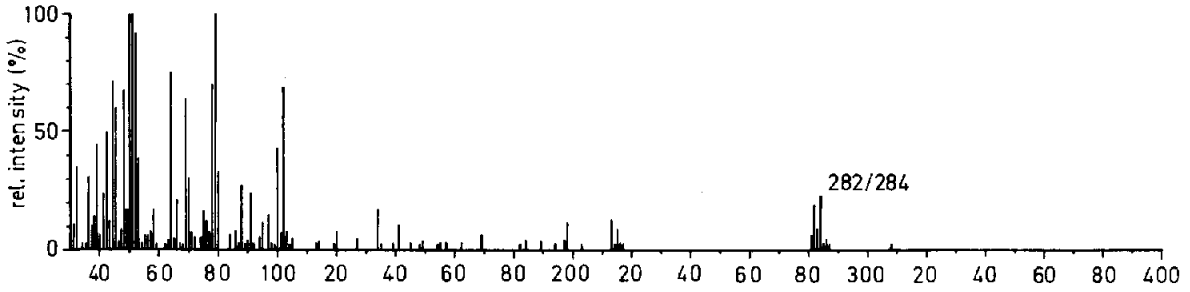

Fig. 1. Electron impact mass spectrum of cysteic acid *p*BrPTH above 30 amu.

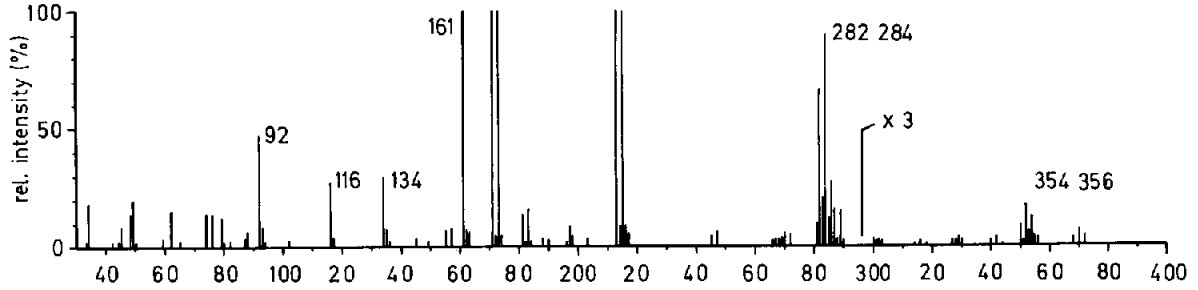

Fig. 2. Electron impact mass spectrum of *S*-Carboxymethyl-cysteine *p*BrPTH above 30 amu.

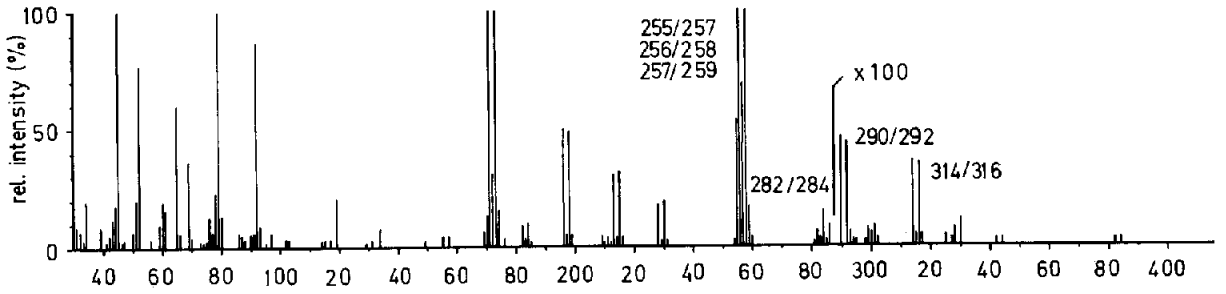

Fig. 3. Electron impact mass spectrum of *S*-Aminoethyl-cysteine *p*BrPTH above 30 amu.

The *S*-aminoethylcysteine *p*BrPTH gives rise to fragments at 255/57, 256/58 (100% rel. abundance), 257/59, 282/84, 290/92 and 314/16. The 255–257 amu fragment series derived from elimination of the ethylamino-p-bromophenyl-thiocarbamyl side chain ($-CH_2CH_2NHCSNHC_6H_4Br$), probably via formation of an amino-thiazolidine derivative undergoing hydrogen abstraction. This predominant series may be used for unequivocal identification of AECys-*p*BrPTH derivatives. Fragmentation of the *S*-ethylamino-*p*-bromophenylthiocarbamyl side chain probably gives rise to the 290/92 amu peak, while positive ionisation of the PTH fragment formed by cleavage of the 257/59 side chain gives rise to the 314/16 peak. The molecular ion peak could not be obtained, fig. 3.

The *S*-vinylpyridyl-*p*BrPTH gives a small molecular ion peak at 421/23 amu, which is too low to be used for identification, fig. 4. However, very intense and

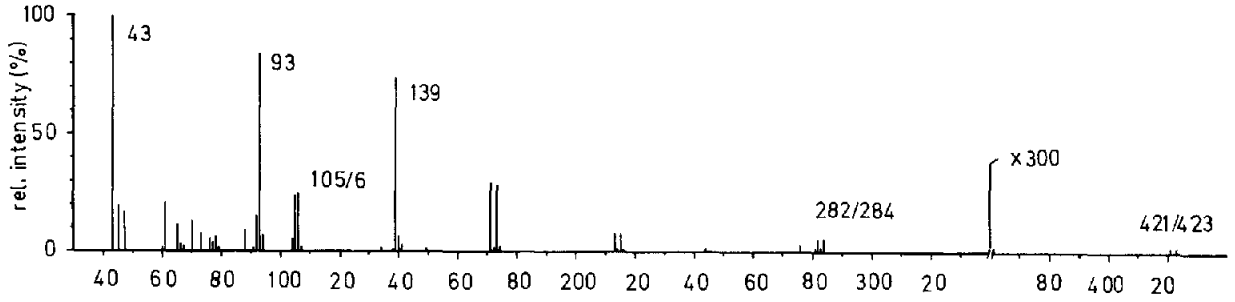

Fig. 4. Electron impact mass spectrum of *S*-Ethylpyridyl-cysteine *p*BrPTH above 30 amu.

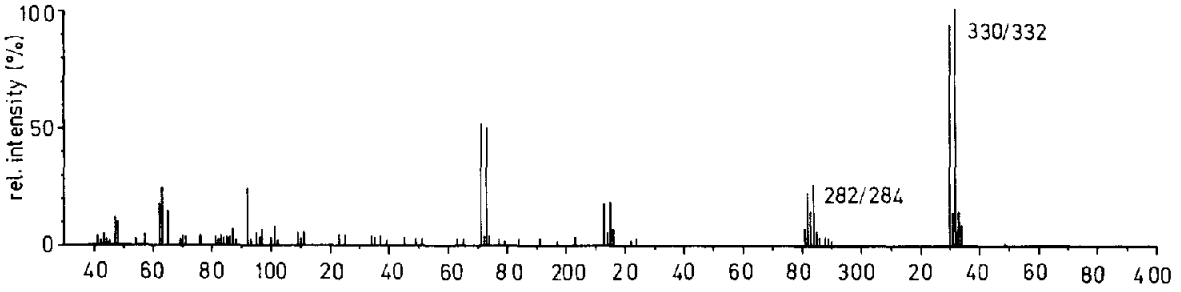

Fig. 5. Electron impact mass spectrum (11 eV) of *S*-Methyl-cysteine *p*BrPTH above 30 amu.

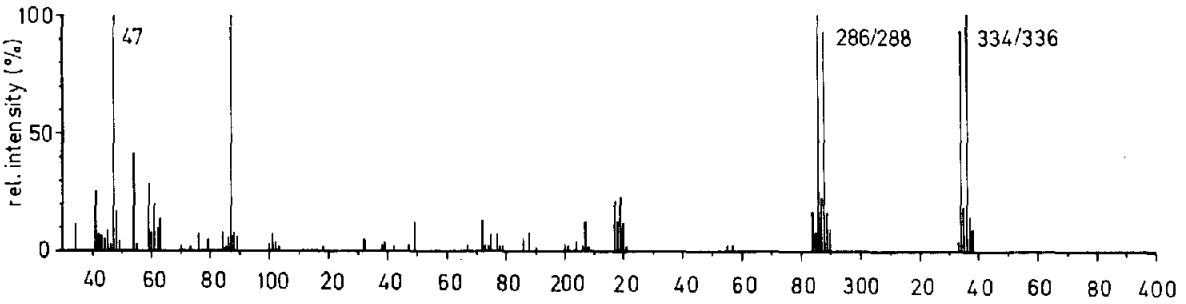

Fig. 6. Electron impact mass spectrum (20 eV) of *S*-Methyl-cysteine *p*Br-d_4-PTH above 30 amu.

characteristic peaks are formed by fragmentation of the *S*-ethylpyridine side chain giving rise to ions at 139 amu and 93 amu.

S-Methylcysteine *p*BrPTH is the derivative best suited for mass spectral identification, fig. 5. The intense molecular ion at 330/32 amu was obtained with 100% rel. abundance. The low molecular weight in comparison to all other derivatives and the non-polar character enhances the volatility of the compound.

At low ionizing voltage (11 eV) the molecular ion is the predominant peak in the mass spectrum. Increasing ionizing voltage (20 eV) gives rise to an increase in 282/84 fragment ions. In this case an intense ion at 47 amu due to cleavage of the *S*-methyl side chain can be detected as is evident from the spectrum of the tetradeuterated *S*-methylcysteine *p*BrPTH.

The deuterated derivatives have been in use in our laboratory for quantification of the mass spectra ob-

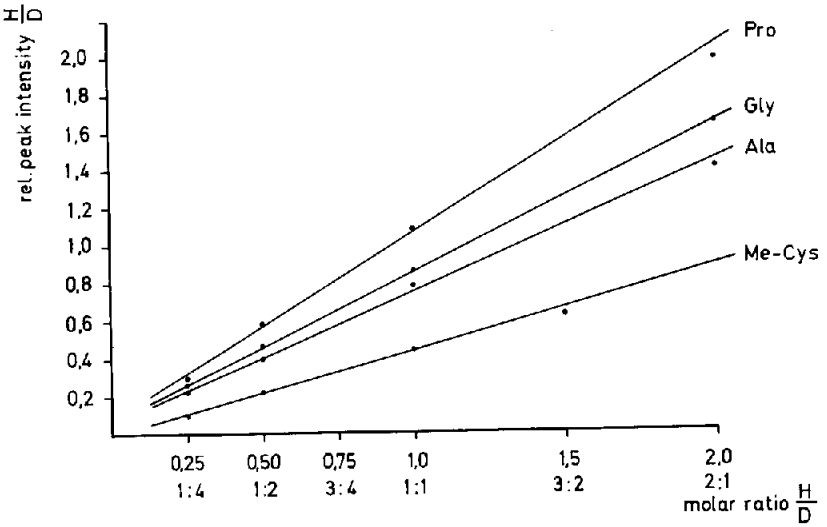

Fig. 7. Diagram demonstrating the exact proportionality between the molar ratio (abscissa) of mixtures of *p*BrPTH derivatives of amino acids and their tetradeuterated analogues and the relative peak intensitics of their corresponding molecular ions (ordinate) in the mass spectrum.

Table 1

Cys Derivative *p*BrPTH	M.W.*	Molecular ion rel. abundance*	Charact. ions (rel. abundance)*
Cys-SO_3H	365	0	282 (19%)
CM-Cys	375	0	354 (4%), 282 (68%), 161 (100%), 134 (30%) 92 (47%).
AE-Cys	574	0	314 (0.1%), 290 (0.5%), 255 (100%), 256 (69%) 257 (19%).
VP-Cys	421	0.01	421 (0.01%), 282 (5%), 139 (74%), 105 (25%) 93 (83%).
Me-Cys	330	100	330 (100%), 282 (24%).
Me-d_4-Cys	334	100	334 (100%).

* Only the ^{79}Br isotope peaks are given.

tained [8]. Mixtures of *p*-bromosubstituted PTH derivatives and their tetradeuterated analogues form ideal solid solutions and the vapour accurately reflects the molar composition of the species. This is demonstrated for some of the common amino acids and their d_4-analogue standards in fig. 7. Exact molar proportionality was obtained between the molar ratios of the undeuterated residue and its deuterated internal standard and the corresponding relative peak intensities in the relevant mass spectra. A general description of this method, which enabled us to determine peptide bond hydrolysis equilibria on a quantitative level [8] will be the subject of a separate paper [22]. ^{15}N-labelled amino acid derivatives have been recommended [23, 24] as well for quantification of the mass spectra of methylthiohydantoines, but this method suffers from the drawback, that the mass of the labeled standard is only 1 amu higher than that of the unlabeled derivative. Overlaps of MTH derivatives differing in 1 amu as in the case of the pairs

Asp/Asn and Glu/Gln (substitution of OH for NH_2) renders interpretation of the mass spectra more difficult. Use of *p*-bromo-substituted phenylisothiocyanate for Edman degradation of proteins [3–9] was found superior to unsubstituted phenylisothiocyanate with respect to 1) more rapid formation of the thiocarbamyl-derivative (coupling reaction) by a factor of 1.7 [22], 2) more easy identification and interpretation of the mass spectra due to the characteristic $^{79/81}Br$ isotope double peak (ratio 50.5 : 49.5) and 3) convenient preparation of a tetradeuterated internal standard with masses of 4 amu higher than the unlabeled derivative. The latter derivatives do not interfere with the identification of other amino acid derivatives. The advent of chemical ionisation techniques [25, 26] is expected to optimize this technique of mass spectral identification of amino acid PTH derivatives with respect to increased total sensitivity by improving the relative intensities of the molecular or quasimolecular ions. First experiments making use of this new technique revealed quite promising results. Preparation of *S*-methylated derivatives of cysteine eliminated the difficulty encountered with the unequivocal identification of this amino acid by mass spectrometry.

Acknowledgements

This investigation was supported by the Deutsche Forschungsgemeinschaft. We wish to thank Miss I. Meloch for her skillful technical assistance in recording of the mass spectra.

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