ACETYLATION AN ARTEFACT IN SOLID PHASE PEPTIDE SYNTHESIS. A MASS SPECTROMETRICAL INVESTIGATION

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1. Introduction

Several authors have demonstrated decreasing coupling yields by increasing chain lengths in solid phase peptide synthesis [1–4]. Particular poor yields were demonstrated in certain coupling steps [2,4].

Perchloric acid titration was used for monitoring of the synthesis of a sequence of antamanid H-Phe––Pro–Pro–Phe–Phe–Val–Pro–Pro–Ala–Phe––resin [4]. A pronounced decrease in the titration value was observed after cleavage of the Boc-group at the tetra, penta, and nonapeptide stage. The overall agreement between the titration values and the amino acid analysis strongly indicated that an inactivation of the amino groups took place in the coupling step.

The mass spectrometrical investigation described here of the above mentioned sequence of antamanid demonstrates that unintentional acetylation of free amino groups had taken place during the synthesis.

2. Experimental

2.1. *Synthesis*

The synthesis by the solid phase method of H-Phe––Pro–Pro–Phe–Phe–Val–Pro–Pro–Ala–Phe-resin was described in [4]. The product was cleaved from the resin by treatment with HBr in CF_3COOH. After removal of the solvent in vacuo at room temp. part of the residue was triturated with ether.

2.2. *Derivatization*

Approx. 1 mg of the crude or the triturated product was dissolved in methanol. HBr bound to the free N-terminal groups was neutralized with a few drops of triethylamine. The free N-terminal amino groups were then deuteroacetylated by addition of hexadeuteroacetic acid anhydride [5], and permethylated with methyliodide using sodium hydride/dimethylsulfoxide as base [6,7].

Samples subjected to the permethylation procedure once are indicated by (A), and samples subjected twice by (B).

2.3. *Mass spectrometry*

The mass spectra were obtained on a Perkin-Elmer 270 mass spectrometer operating at 70 eV. The samples were introduced directly into the ion source and the temperature of the solids inlet probe was slowly increased from 70° to 300°. During this period a spectrum was recorded for each 20° to 30° increase in temperature in order to observe possible fractionated vaporization. The temperature of the ion source was 150°.

The elemental composition of some characteristic ions was determined by high resolution mass measurements (± 3 ppm) on an AEI MS 902 mass spectrometer.

3. Results

The mass spectra of the crude and the ether triturated products showed that no N-methylation had

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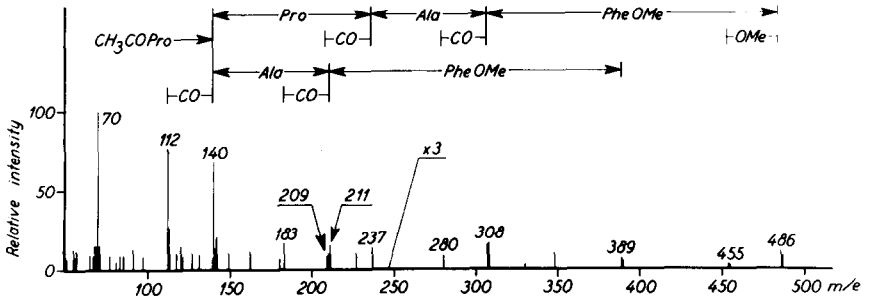

Fig. 1. Mass spectrum of a non N-methylated sample (A). Solids inlet temp 170°.

taken place when the permethylation procedure was carried out only once (A). Several acylated and esterified peptides were however observed.

Repetition of the permethylation procedure resulted in complete N-methylation (B). The permethylated peptides, however, showed two types of anomalities. So N-terminal acetyl proline residues were partly methylated, whereas proline residues in the peptide chain were not methylated. Furthermore C-terminal methylesters could not be demonstrated. Intense peaks, however, were found corresponding to the expected molecular ions −17, which is equal to the largest acyl peptide fragment +14. Both anomalities mentioned have occasionally been found in our laboratory by permethylation of peptides. The methylation of N-terminal proline residues, showed to be an advantage for the interpretation. This because all sequence peaks belonging to peptides with N-terminal proline appeared as doublets 14 mass units apart. The lack of molecular peaks in the spectra of (B) caused no difficulties for the interpretation as intense molecular peaks appeared in the spectra of (A).

Two N-terminal acylated amino acids, proline and phenylalanine were found by the interpretation of the spectra. Thus acetyl proline indicated by peaks at 140 $CH_3CO-\overline{N-CH_2-CH_2-CH_2-CH}-CO^+$, and 112, $CH_3CO-\overline{N-CH_2-CH_2-CH_2-CH}^+$, were present in the spectra obtained at all temperatures of (A) as well as of (B). In (B) peaks were furthermore found at 126 and 154 corresponding to the above mentioned methylation of the acetyl proline residue. High resolution measurements of these ions demonstrated the elemental compositions: $C_7H_{10}O_2N$ (m/e 140), $C_6H_{10}ON$ (m/e 112) and $C_8H_{12}O_2N$ (m/e 154). The presence of acetylated respectively acetylated and methylated proline thus were confirmed. In the spectra obtained from 240° to 300° deuteroacetyl-phenylalanine could be demonstrated. This was indicated by peaks at 194 and 165 in the spectra of (A) and by peaks at 207 and 179 in the spectra of (B). Furthermore a small amount of N-terminal acetyl phenylalanine was indicated by peaks at 204 and 176 in the spectra obtained at 260° of (B). The peak at 194 of the deuteroacetyl-phenylalanine residue in the spectra

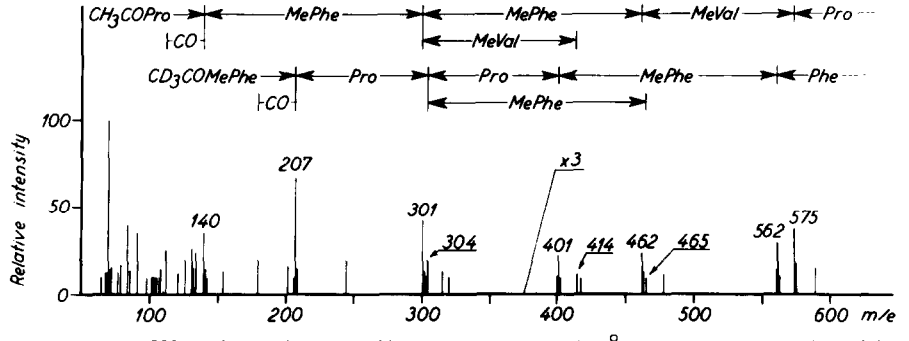

Fig. 2. Part of the mass spectrum of N-methylated sample (B). Solids inlet temp 260°. The peaks in table 2, which are indicated as very weak, are not considered in the figure.

Table 1
Sequences deduced from mass spectra of non N-methylated samples (A).

I	160–180°	CH_3CO–Pro	[140]	(112)–Ala	[211]	(183)–PheOMe	[389]												
II	160–215°	CH_3CO–Pro	[140]	(112)–Pro	[237]	(209)–Ala		[308]	(280)–Phe	[455]	(427)–OMe	[486]							
III	250–270°	CH_3CO–Pro	[140]	(112)–Phe	[287]	(259)–Phe		[434]	(406)–Val	[533]	(505)–Pro		[630]	(602)–					
IV	250–270°	CD_3CO–Phe	[194]*	(165)–Pro	[290]	–Pro		[387]	–Phe	[534]	(506)–Phe		[681]	(653)–Val		[780]§–			
V	250–270°	CH_3CO–Pro	[140]	(112)–Phe	[287]	(259)–Val		[386]	(358)–Pro	[483]	§–								

The sequences I–III represent truncated acetylated peptides, IV the deuteroacetylated decapeptide and V peptide with failure sequence. The first column indicates the temperature of the solids inlet probe. The m/e of the sequence peaks corresponding to the fragment ions are indicated in [ ], and m/e of the peaks corresponding to the loss of CO from the sequence ions is indicated, when present, in ( ).

* This peak was expected at m/e 193 but was found at m/e 194.
§ Very weak peaks.

Table 2
Sequences deduced from mass spectra of N-methylated samples (B).

I	140–170°	CH_3CO–Pro	[140]*	(112)–MeAla	[225]*	–MePhe	[386]*†												
II	170–215°	CH_3CO–Pro	[140]*	(112)–Pro	[237]*	–MeAla	[322]*	–MePhe	[483]*†										
III	240–280°	CH_3CO–Pro	[140]*	(112)–MePhe	[301]*	–MePhe	[462]*	–MeVal	[575]*	–Pro	[672]*	–Pro	[769]*	–MeAla	[854]*	–			
IV	260°	CH_3CO–MePhe	[204]	§(176)–MePhe	[365]§	–MeVal	[478]§–												
V	260–280°	CD_3CO–MePhe	[207]	(179)–Pro	[304]	–Pro	[401]	MePhe	[562]	–MePhe	[723]	–MeVal	[836]	–Pro	[933]	–			
VI	240–280°	CH_3CO–Pro	[140]*	(112)–MePhe	[301]*	–MeVal	[414]*	–Pro	[511]§–										
VII	260–280°	CH_3CO–Pro	[140]*	(112)–MePhe	[301]*	–MePhe	[462]*	–MeVal	[575]*	–Pro	[672]*	–MeAla	[757]	–					
VIII	260–280°	CD_3CO–MePhe	[207]	(179)–Pro	[304]	–MePhe	[465]	–MePhe	[626]§	–MeVal	[739]	–							

The sequences I–IV represent truncated acetylated peptides. V the deuteroacetylated decapeptide and VI–VIII peptides containing failure sequences. The first column indicates the temperature of the solids inlet probe. The m/e of the sequence peaks corresponding to the fragment ions are indicated in [ ], and the m/e of the peaks corresponding to loss of CO from the sequence ions are indicated, when present, in ( ).

* The peak is followed by a peak 14 mass units higher due to methylated N-terminal proline.
† Anomal C-terminal.
§ Very small peak.

of (A) was expected to appear at m/e 193. However, a peak at 165, which is loss of CO from 193, and a peak at 290 corresponding to deuteroacetyl Phe–Pro was found.

The results presented in the figures and tables were obtained with the ether triturated samples. The non-triturated samples contained considerable amounts of non-peptide material making them less suitable for a mass spectrometrical investigation. The sequences, which could be deduced from the spectra of (A), are shown in table 1 and of (B) in table 2. The results are based on duplicate preparations. The spectrum obtained at 170° of (A) and part of the spectrum obtained at 260° of (B) are shown in figs. 1 and 2, respectively.

4. Discussion

The demonstration of N-terminal acetylproline and small amounts of acetylphenylalanine besides the expected N-terminal deuteroacetyl-phenylalanine shows that an acetylation of α-amino groups has taken place during the synthetic procedure. The sequences deduced from the possible N-terminals, furthermore show that the acetylation mainly has taken place at the tri, tetra, and octa peptide stage.

As mentioned in the introduction the perchloric acid titration values obtained during the synthesis demonstrated a pronounced decrease in the amount of free amino groups after the removal of the Boc-group at the tetra, penta and nona peptide stage [4]. This shows that the acetylation must have occurred during the coupling step, as otherwise a decrease in the titration value already would have been observed after the removal of the Boc-group at the tri, tetra, and octa peptide stage. This conclusion was further supported by the agreement between the amino acid content of the synthetic products as determined by amino acid analysis and calculated from the titration values [4]. The failure sequences in tables 1 and 2 were based upon very weak peaks in the mass spectra. These peaks, however, were reproducible and are therefore indicative of the presence of small amounts of failure sequences. It deserves notice that the failure sequences found, are missing a proline or phenylalanine residue in the positions where Pro–Pro or Phe–Phe were expected. This may simply be due to the fact that the same failure sequences can be caused by the absence of one or the other of the identical residues.

Considering only the spectra of (B) the methylation of N-terminal acetyl proline may lead to the interpretation of acetylation as well as of propionylation. The mass spectra of (A), however, shows that acetylation but not propionylation had taken place, because of the absence in these spectra of peaks at m/e 154 and 126. In the spectra of (B) the amine fragment of proline at m/e 70 was followed by a peak at m/e 84, which might indicate that the extra methyl group was introduced in the ring system of the proline residue. The ion at m/e 84, however, might also have been formed by rearrangement involving a transfer of a methyl group substituted in the acetyl group by the permethylation procedure. This could be the explanation of the fact that only N-terminal proline was found to be methylated.

The limited mass range and sensitivity of the mass spectrometer used, is the reason why it has not been possible to observe fragment ions at masses greater that approx. 1000. This has, however, not been a serious limitation in this experiment. A comparison between the results obtained with (A) and (B) shows that it was, as expected, possible to detect sequence peaks of larger fragments, when the sample was N-methylated.

Exact quantitative determination of the amount of the different peptides is not possible from the mass spectral data. However, the combination with the titration values results in information of the nature as well as of the amount of blocking.

The acetylation of the peptides can be explained by the presence of acetic acid during the coupling step. In [4] it was demonstrated by the use of ^{14}C-HOAc that the resin and especially the teflon parts of the reaction system absorbed sufficient amounts of HOAc to explain the degree of acetylation. In the reaction system teflon was used for part of the reactor, for the tubings and the membrane circulation pump.

Mass spectrometrical investigations of a crude product obtained by solid phase peptide synthesis have thus proved to be a useful supplement to other analytical methods. The capability of mass spectrometry for analyzing peptide mixtures has been described in [5,8–10]. In the present investigation sequential information has been obtained of 8 peptides in a mix-

ture and furthermore an undesired amino blocking group was identified.

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