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AMINO-ACID SEQUENCE STUDY IN PEPTIDES BY MASS SPECTROMETRY—II*

INVESTIGATION OF PENTADEUTEROBENZOYL-PEPTIDE METHYL ESTERS

J. P. KAMERLING, W. HEERMA, TH. J. PENDERS and J. F. G. VLIEGENTHART
Laboratories of Organic Chemistry and Analytical Chemistry,
University of Utrecht, The Netherlands

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Abstract—The utility of benzoyl and pentadeuterobenzoyl derivatives of peptide methyl esters for mass spectrometric analysis was investigated. The mass spectra of the glu-his and the val-tyr-pro derivatives are discussed. Treatment of the peptide methyl esters with the mixed benzoic-ethylcarbonic anhydride did in some cases lead to benzoyl derivatives as well as to ethoxycarbonyl derivatives.

INTRODUCTION

IN THE course of mass spectrometric investigations of the amino-acid sequence of peptides,[1,2,3] we recently reported how the detection of C-terminal fragments is facilitated by comparing the mass spectra of methyl esters with those of trideuteromethyl esters.[3] As, however, the number of N-terminal fragments is usually more abundant than that of C-terminal fragments, the importance of the immediate indication of the N-terminal fragments is evident.[4] To that purpose we decided to use the benzoyl and pentadeuterobenzoyl components. Fragments of a peptide derivative containing one pentadeuterobenzoyl group will show a shift of 5 mass units respective to the corresponding benzoyl fragments.

In the course of the experimental elaboration of this principle we found that sometimes during the preparation of the benzoyl derivatives by-products are formed. The tracing of these by-products was also greatly facilitated by the application of the pentadeuterobenzoyl group.

RESULTS AND DISCUSSION

The mass spectra of glutamylhistidine methyl ester converted with benzoic-ethylcarbonic anhydride (Fig. 1a) and with pentadeuterobenzoic-ethylcarbonic anhydride (Fig. 1b) were recorded at 140°C. Molecular peaks corresponding to the bis-benzoyl product at *m/e* 520 or to the bis-pentadeuterobenzoyl product were not present in the spectra. Comparison of the spectra showed, that several high mass peaks which should still contain the N-terminal fragment did not shift. The conclusion must be that the samples consisted of mixtures of products, although no inhomogeneity was observed by thin-layer chromatography. By exact mass measurements (Table 1) the products were identified as benzoylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidine methyl ester (Scheme 1a), as ethoxycarbonylglutamyl(methyl ester)-(im-N-ethoxycarbonylhistidine methyl ester (Scheme 1b). No indication could be found that the partial introduction of one or two ethoxycarbonyl groups instead of benzoyl groups was caused by the presence of unchanged ethylchlorocarbonate. In the literature other examples of the partial introduction of an ethoxycarbonyl group

* For Part I see Ref. 3.

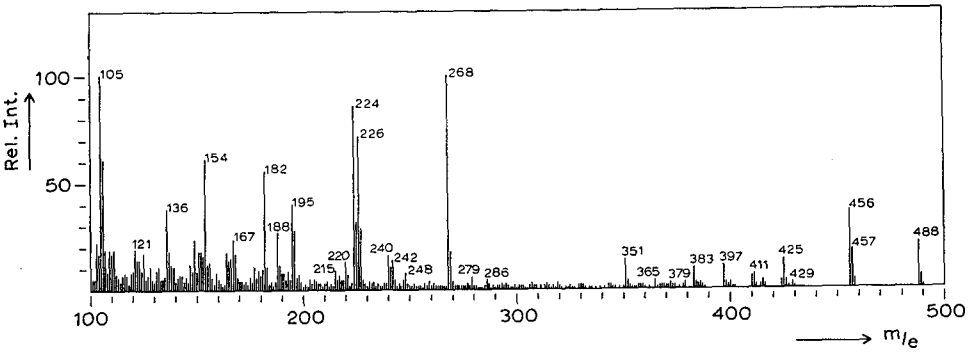


FIG. 1a. Mass spectrum of a mixture of benzoylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidine methyl ester and ethoxycarbonylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidine methyl ester obtained by means of the mixed anhydride method. Only *m/e* values > 100 are given.

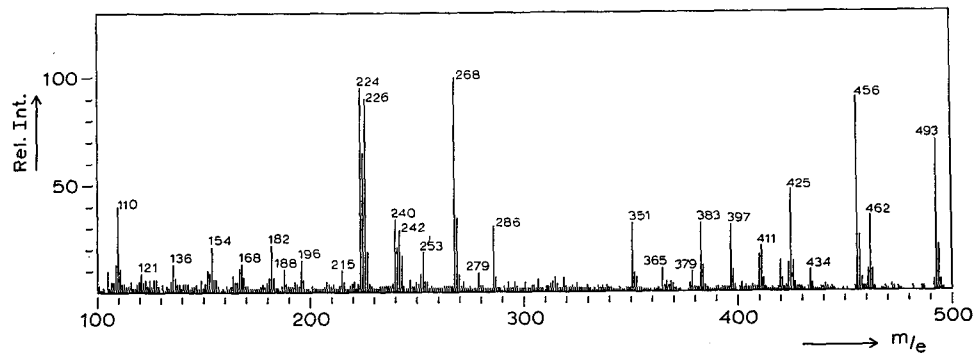
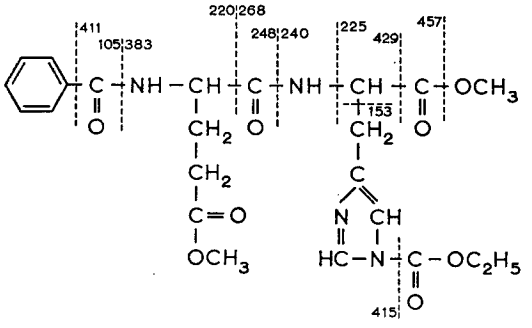


FIG. 1b. Mass spectrum of a mixture of pentadeuterobenzoylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidine methyl ester and ethoxycarbonylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidine methyl ester obtained by means of the mixed anhydride method. Only *m/e* values > 100 are given.

TABLE 1. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF THE MIXTURE OF GLUTAMYLHISTIDINE DERIVATIVES (SCHEME 1a + 1b)

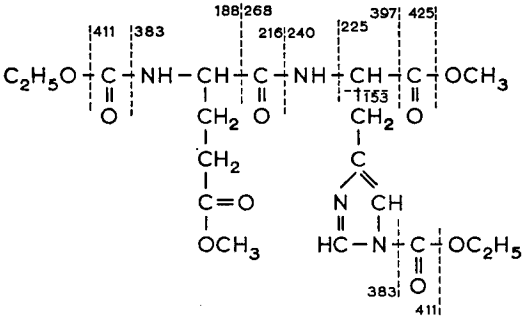
m/e	Measured value	Calculated value	Empirical formula
224	224·0799	224·0797	$C_{10}H_{12}N_2O_4$
226	226·0952	226·0954	$C_{10}H_{14}N_2O_4$
240	240·0989	240·0984	$C_{10}H_{14}N_3O_4$
242	242·1140	242·1141	$C_{10}H_{16}N_3O_4$
268	268·0934	268·0933	$C_{11}H_{14}N_3O_5$
286	286·1166	286·1165	$C_{12}H_{18}N_2O_6$
397	397·1717	397·1723	$C_{17}H_{25}N_4O_7$
425	425·1661	425·1672	$C_{18}H_{25}N_4O_8$
456	456·1831	456·1856	$C_{19}H_{28}N_4O_9$
488	488·1910	488·1907	$C_{23}H_{28}N_4O_8$

M = 488



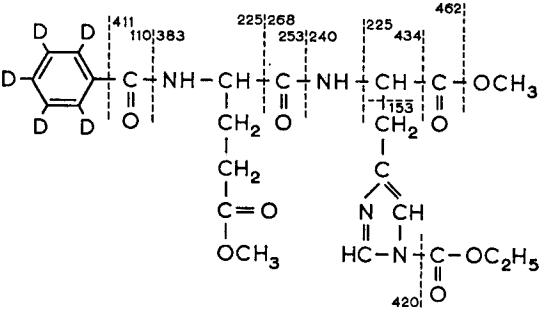
SCHEME 1a. Structure of benzoylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidine methyl ester and its fragmentation pattern.

M = 456



SCHEME 1b. Structure of ethoxycarbonylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)-histidine methyl ester and its fragmentation pattern.

M = 493



SCHEME 1c. Structure of pentadeuterobenzoylglutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidine methyl ester and its fragmentation pattern.

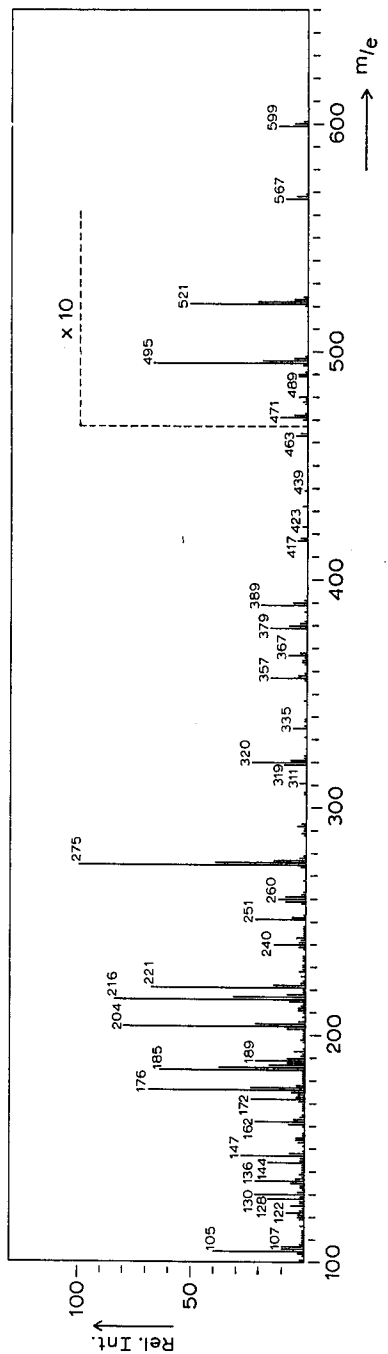


FIG. 2a. Mass spectrum of benzoylvalyl-(O-benzoyl)tyrosylproline methyl ester polluted with by-products, as indicated in the text. Only *m/e* values > 100 are given.

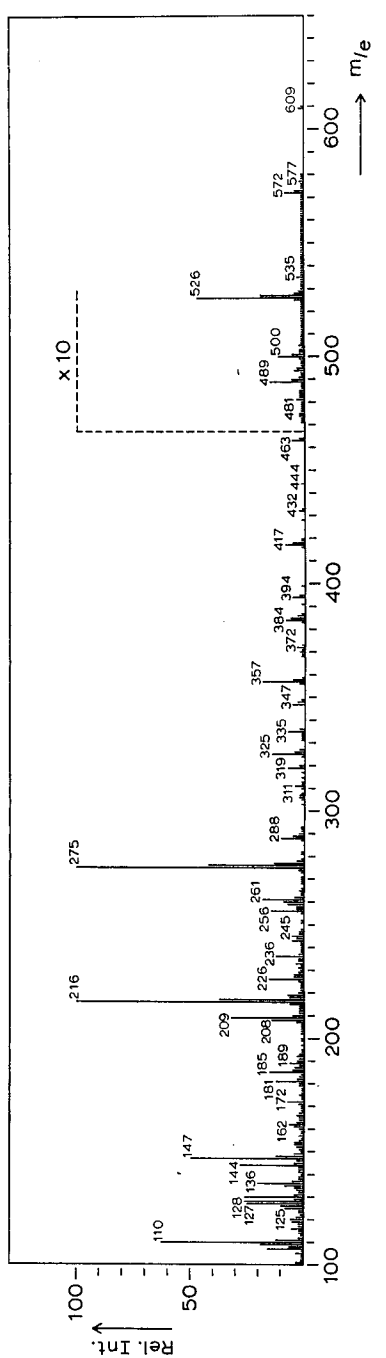
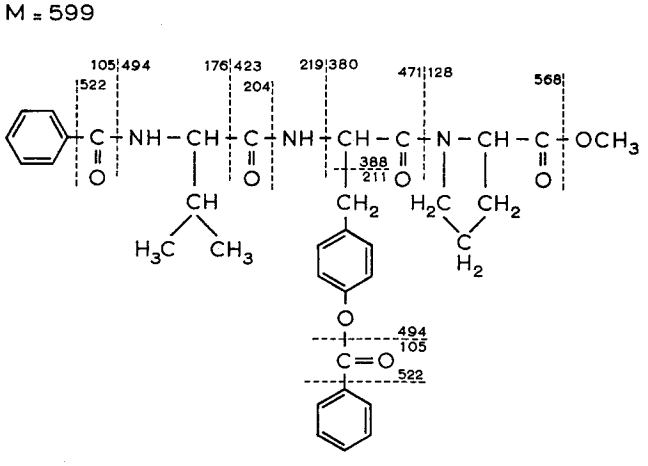


FIG. 2b. Mass spectrum of pentadeuterobenzoylvalyl-(O-pentadeuterobenzoyl)tyrosylproline methyl ester, polluted with by-products, as indicated in the text. Only *m/e* values > 100 are given.

to the N-terminal side of a peptide are known[5,6,7] by application of the mixed anhydride method. The attachment of the ethoxycarbonyl group of the mixed anhydride to the imidazole ring of histidine seems to be rather general, as we also observed this for glutamyl(methyl ester)-histidylphenylalanine methyl ester and for histidine methyl ester; no im-N-benzoyl products could be detected. It is noteworthy, that the volatility of the ethoxycarbonyl derivative is higher than of the benzoyl derivative. In order to avoid these complications we tried to prepare the



SCHEME 2. Structure of benzoylvalyl-(O-benzoyl)tyrosylproline methyl ester and its fragmentation pattern.

benzoyl derivatives by means of the symmetric anhydride; however, this method again yielded a mixture of two products, notably: benzoylglutamyl(methyl ester)-(im-N-benzoyl)histidine methyl ester and benzoylglutamyl(methyl ester)-histidine methyl ester. The first derivative was formed in a small amount only and did not cause troubles in the interpretation of the mass spectrum.

The fragmentation pattern of benzoylvalyl-(O-benzoyl)tyrosylproline methyl ester (Fig. 2a and Scheme 2) prepared by the mixed anhydride method is rather similar to that of the caproyl derivative.[3] Fragment ions of pentadeuterobenzoylvalyl-(O-pentadeuterobenzoyl)tyrosylproline methyl ester containing two pentadeuterobenzoyl groups give a shift of 10 mass units, when compared with the same non-deutero compound (Fig. 2b). A shift of 5 mass units does not necessarily point to an N terminal fragment. The mass spectrum was recorded at 190°C. The volatility of the benzoyl derivative is comparable to that of the caproyl compound.

In these spectra too some peaks were present, which belong to ethoxycarbonyl derivatives. By exact mass measurements (Table 2) these by-products were identified as benzoylvalyl-(O-ethoxycarbonyl)tyrosylproline methyl ester, ethoxycarbonylvalyl-(-O-benzoyl)tyrosylproline methyl ester, and ethoxycarbonylvalyl-(O-ethoxycarbonyl)tyrosylproline methyl ester. Again these by-products have a greater volatility than the benzoyl derivatives.

The relatively high volatility of the ethoxycarbonyl compounds, and the rather intense molecular and other peaks which we found in the mass spectra, made it attractive

TABLE 2. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF THE MIXTURE OF VALYLTYROSYLPROLINE DERIVATIVES

m/e	Measured value	Calculated value	Empirical formula
144	144·1016	144·1024	$C_7H_{14}NO_2$
161	161·0489	161·0477	$C_9H_7NO_2$
162	162·0550	162·0555	$C_9H_8NO_2$
162	162·0918	162·0919	$C_{10}H_{12}NO$
172	172·0975	172·0974	$C_8H_{14}NO_3$
185	185·0930	185·0926	$C_8H_{13}N_2O_3$
221	221·1301	221·1290	$C_{12}H_{17}N_2O_2$
389	389·1955	389·1951	$C_{20}H_{27}N_3O_5$
439	439·1889	439·1869	$C_{24}H_{27}N_2O_6$
463	463·2331	463·2318	$C_{23}H_{33}N_3O_7$
521	521·2167	521·2162	$C_{28}H_{31}N_3O_7$
599	599·2618	599·2631	$C_{34}H_{37}N_3O_7$

to see whether protection of peptides with such a group was useful for mass spectrometric analysis. In the following paper we report on the very special advantages offered by ethoxycarbonyl protection.

EXPERIMENTAL

Pentadeuterobenzoic acid was prepared from hexadeuterobenzene (Merck) via pentadeuterobromobenzene.[8,9]

The peptides were converted into the corresponding methyl esters by reaction with methanol saturated with hydrogen chloride.[10] Acylation of the peptide methyl esters was performed by means of benzoic acid or pentadeuterobenzoic acid by using the mixed anhydride method[11]. The mixed anhydride was formed by reaction of the mentioned acids and ethylchlorocarbonate.

The peptide derivatives were purified by preparative thin-layer chromatography using Kieselgel G (Merck) and the solvent system petroleum ether (boiling range 40–60°C): diethylether = 15:20. The spots of a parallel run were developed with Cl_2/*o*-tolidine-KI.[12]

70 eV mass spectra were recorded with a MS-9 mass spectrometer (AEI).

REFERENCES

1. Th J. Penders, H. Copier, W. Heerma, G. Dijkstra and J. F. Arens, *Rec. Trav. Chim.* **85**, 216 (1966).
2. Th J. Penders, H. Copier, W. Heema, G. Dijkstra and J. F. Arens, *Rec. Trav. Chim* **85**, 879 (1966).
3. J. P. Flikweert, W. Heerma, Th. J. Penders, G. Dijkstra and J. F. Arens, *Rec. Trav. Chim.* **86**, 293 (1967).
4. J. v. Heyenoort, E. Bricas, B. C. Das, E. Lederer, and W. A. Wolstenholme, *Tetrahedron* **23**, 3403 (1967).
5. L. Gatterman and Th. Wieland, *Die Praxis des organischen Chemikers*, W. de Gruyter, Berlin 1961, p. 95.
6. A. S. Hussey, *J. Am. Chem. Soc.* **73**, 1365 (1951).
7. E. J. Wilson and E. Pascu, *J. Org. Chem.* **7**, 126 (1942).
8. E. Bricas, J. v. Heyenoort, M. Barber, W. A. Wolstenholme, B. C. Das and E. Lederer, *Biochemistry* **4**, 2254 (1965).
9. F. Reindel and U. W. Hoppe, *Chem. Ber.* **87**, 1103 (1954).
10. F. E. King, J. W. Clark-Lewis, D. A. A. Kidd and G. R. Smith, *J. Chem. Soc.* **1039** (1954).
11. A. R. Batterby and J. C. Robinson, *J. Chem. Soc.* 259 (1955).
12. D. Gilessen, E. Schnabel and J. Meienhofer, *Ann. Chem.* **667**, 164 (1963).