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

INVESTIGATION OF ETHOXYCARBONYL-PEPTIDE METHYL ESTERS

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Abstract—The utility of ethoxycarbonyl-peptide methyl esters for mass spectrometric analysis is described. The ethoxycarbonyl group has important advantages over other protecting groups which are in use. The derivatives have high volatility and in the mass spectrum the molecular and the sequence peaks have relatively high intensity; this greatly facilitates the interpretation of the fragmentation pattern. The spectra of the methyl esters of the ethoxycarbonyl derivatives of pro-val, trp-gly, gly-ser, $(cys)_2$, glutathion (GSH), glu-his-phe, val-tyr-pro and val-lys-val-tyr-pro are given.

INTRODUCTION

THE application of the mass spectrometric determination of the amino-acid sequence in polypeptides is still restricted to small peptides. The largest peptides investigated so far are some natural peptidolipids[1] containing 9 amino-acid residues. One of the limiting factors is the low volatility of the peptides. This can be improved by application of protecting groups which diminish the extent of intermolecular hydrogen bonding. The carboxylic groups are usually converted into methyl esters, while the amino group and other functional groups in the amino-acid residues are acylated or arylated.[2–10] The volatility of the peptide derivatives is dependent on the protecting groups, although other factors also play an important rôle, e.g. intermolecular hydrogen bonding[5,11] due to the peptide bonds.

Interpretation of a mass spectrum is highly facilitated when the N-protecting group stabilizes the molecule and reduces the complexity of the fragmentation pattern. Preferably the amino-acid sequence peaks should be easily recognizable. During previous work[12] we observed the relatively high volatility of ethoxycarbonyl-peptide methyl esters. For this reason we studied the suitability of such peptide derivatives for mass spectrometric analysis and it will be shown in this paper that ethoxycarbonyl protection of functional groups has very distinct advantages over other methods.

RESULTS

I. *Ethoxycarbonylprolylvaline methyl ester*

Molecular formula: $C_{14}H_{24}N_2O_5$. Ion source temperature 100°C. The data are presented in Fig. 1, Scheme 1 and Table 1a and 1b.

* In Part II see preceding paper.

II. *Ethoxycarbonyltryptophylglycine methyl ester*

Molecular formula: $C_{17}H_{21}N_3O_5$. Ion source temperature: 120°C. The data are presented in Fig. 2, Scheme 2 and Table 2a and 2b. The degradation of the tryptophane side chain is apparent from the series *m/e* 130 → *m/e* 103 + HCN → *m/e* 77 + HC≡CH.[13]

III. *Ethoxycarbonylglycylserine methyl ester*

Molecular formula $C_9H_{16}N_2O_6$. Ion source temperature: 120°C. The data are presented in Fig. 3, Scheme 3 and Table 3. No molecular peak was observed. The highest *m/e* value (230) is the result of water elimination.

IV. *Bisethoxycarbonylcystine bismethyl ester*

Molecular formula $C_{14}H_{24}N_2O_8S_2$. Ion source temperature: 75°C. The data are presented in Fig. 4, Scheme 4 and Table 4a and 4b. Although the intensity of the molecular peak is about 35% of the base peak, almost all fragmentations with a relatively large intensity arise from cleavages in or near the disulfide bridge.

V. *Ethoxycarbonyl-γ-glutamyl(methyl ester)-(S-ethoxycarbonyl)cysteinylglycine methyl ester*

Molecular formula $C_{18}H_{29}N_3O_{10}S$. Ion source temperature: 150°C. The data are presented in Fig. 5, Scheme 5 and Table 5.

VI. *Ethoxycarbonyl-α-glutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidylphenylalanine methyl ester*

Molecular formula $C_{28}H_{37}N_5O_{10}$. Ion source temperature: 160°C. The data are presented in Fig. 6, Scheme 6 and Table 6. Note that a product is present with a peak which is 27 mass units higher than the parent peak of the peptide derivative.

VII. *Ethoxycarbonylvalyl-(O-ethoxycarbonyl)tyrosylproline methyl ester*

Molecular formula $C_{26}H_{37}N_3O_9$. Ion source temperature: 140°C. The data are presented in Fig. 7, Scheme 7, and Table 7a and 7b. In the logarithmic intensity spectrum peaks were present related to the product with molecular-formula $C_{27}H_{35}N_3O_{10}$ (≡peptide derivative plus CO, minus 2H). The corresponding *m/e* values of 561 and 458 are omitted in Fig. 7 because their intensity was less than 1%.

VIII. *Ethoxycarbonylvalyl-(N-ethoxycarbonyl)lysylvalyl-(O-ethoxycarbonyl)-tyrosylproline methyl ester*

Molecular formula $C_{40}H_{62}N_6O_{13}$. Ion source temperature: 210°C. The data are presented in Fig. 8, Scheme 8 and Table 8. Note again that a product is present with a molecular peak which is 26 mass units higher than the molecular peak of the peptide derivative.

DISCUSSION

In this investigation the carboxylic groups were always converted into methyl esters. The application of other esters seems to be an attractive possibility to obtain

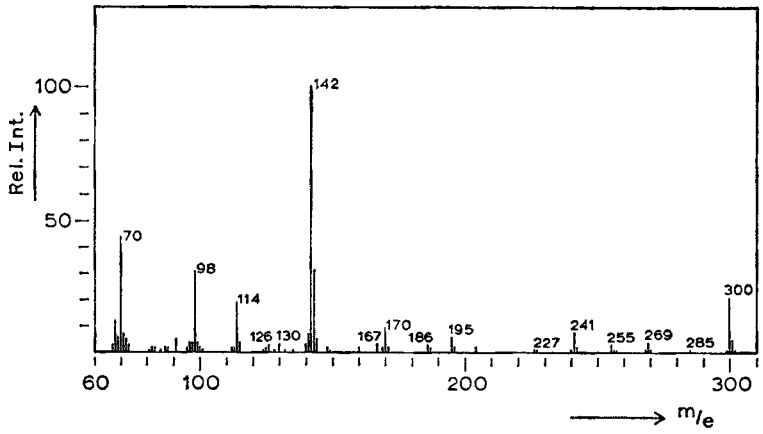
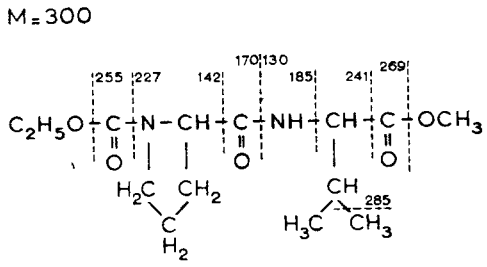


FIG. 1. Mass spectrum of ethoxycarbonylprolylvaline methyl ester. Only *m/e* values >60 are given.



SCHEME 1. Structure of ethoxycarbonylprolylvaline methyl ester, and its fragmentation pattern.

TABLE 1a. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF ETHOXYCARBONYLPROLYLVALINE METHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
70	70·0657	70·0657	C_4H_8N	$142 - COOC_2H_5 + H$
114	114·0550	114·0555	$C_5H_8NO_2$	$142 - C_2H_4$
142	142·0862	142·0868	$C_7H_{12}NO_2$	see structure
300	300·1692	300·1685	$C_{14}H_{24}N_2O_5$	see structure

TABLE 1b. INTERPRETATION OF METASTABLE PEAKS IN THE MASS SPECTRUM OF ETHOXYCARBONYLPROLYLVALINE METHYL ESTER

*m**	$m_1^+ \rightarrow m_2^+$	Eliminated group
216·7	300 → 255	C_2H_5O
215·9	269 → 241	CO
119.9	241 → 170	$NHCH[CH(CH_3)_2]$
118·6	170 → 142	CO
91·5	142 → 114	C_2H_4
67·6	142 → 98	CH_3CHO
50·0	98 → 70	CO

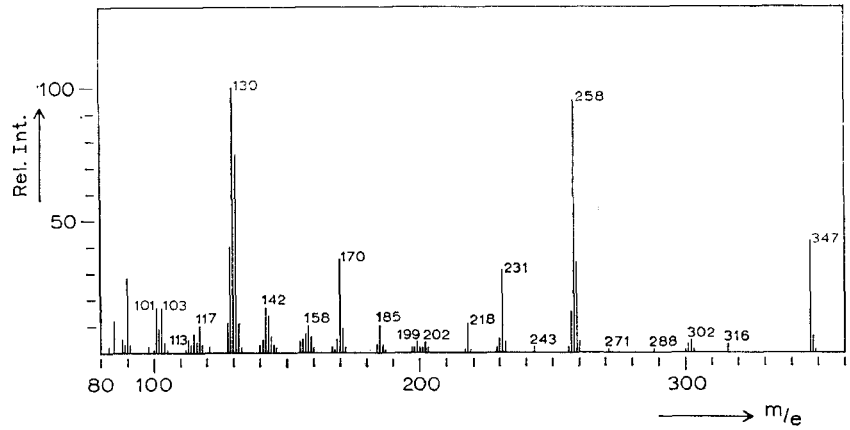
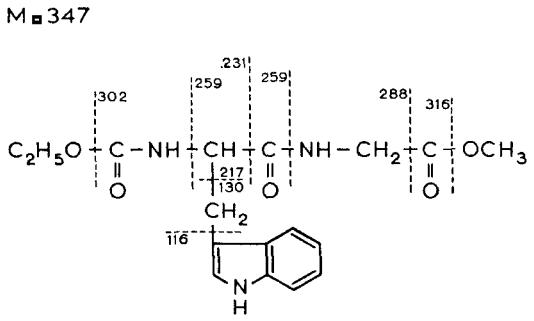


FIG. 2. Mass spectrum of ethoxycarbonyltryptophylglycine methyl ester. Only *m/e* values >100 are given.



SCHEME 2. Structure of ethoxycarbonyltryptophylglycine methyl ester, and its fragmentation pattern.

TABLE 2a. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF ETHOXYCARBONYLTRYPTOPHYLGLYCINE METHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
103	103·0543	103·0548	C_8H_7	130 − HCN
117	117·0581	117·0578	C_8H_7N	116 + H
130	130·0662	130·0657	C_9H_8N	see structure
170	170·0605	170·0606	$C_{11}H_8NO$	259 − $NHCOOC_2H_5$ − H
202	202·0755	202·0742	$C_{11}H_{10}N_2O_2$	231 − C_2H_5
231	231·1128	231·1133	$C_{13}H_{15}N_2O_2$	see structure
258	258·1006	258·1004	$C_{14}H_{14}N_2O_3$	259 − H
347	347·1472	347·1481	$C_{17}H_{21}N_3O_5$	see structure

TABLE 2b. INTERPRETATION OF METASTABLE PEAKS IN THE MASS SPECTRUM OF ETHOXYCARBONYLTRYPTOPHYLGLYCINE METHYL ESTER

*m**	$m_1^+ \rightarrow m_2^+$	Eliminated group
191·8	347 → 258	$NH_2COOC_2H_5$
153·8	347 → 231	$CONHCH_2COOCH_3$
81·6	130 → 103	C_2H_3
57·6	103 → 77	HC≡CH

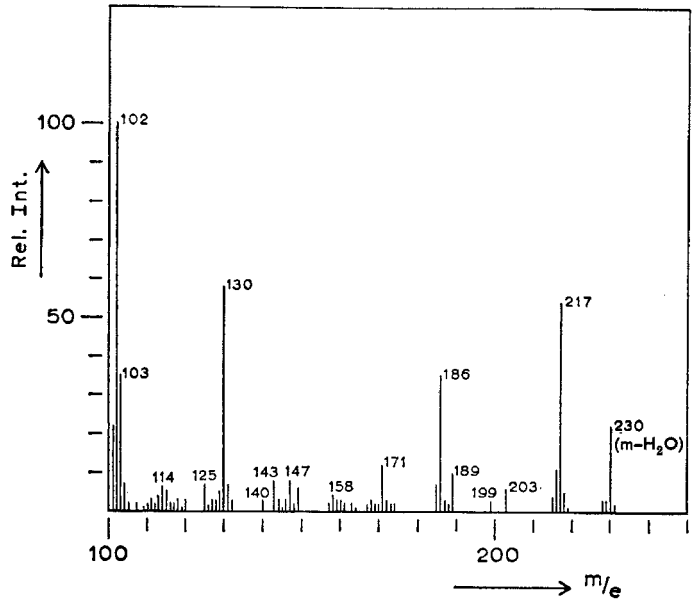
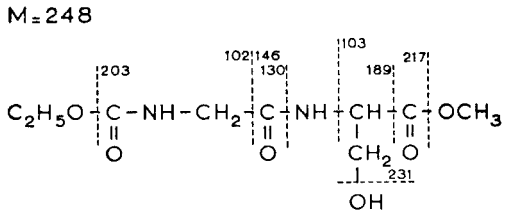


FIG. 3. Mass spectrum of ethoxycarbonylglycylserine methyl ester. Only *m/e* values >100 are given.



SCHEME 3. Structure of ethoxycarbonylglycylserine methyl ester, and its fragmentation pattern.

TABLE 3. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF ETHOXYCARBONYLGLYCYLSERINE METHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
125	125·0345	125·0351	$C_5H_5N_2O_2$	231 − $COOCH_3$ − OC_2H_5 − 2H
230	230·0880	230·0903	$C_9H_{14}N_2O_5$	M − H_2O

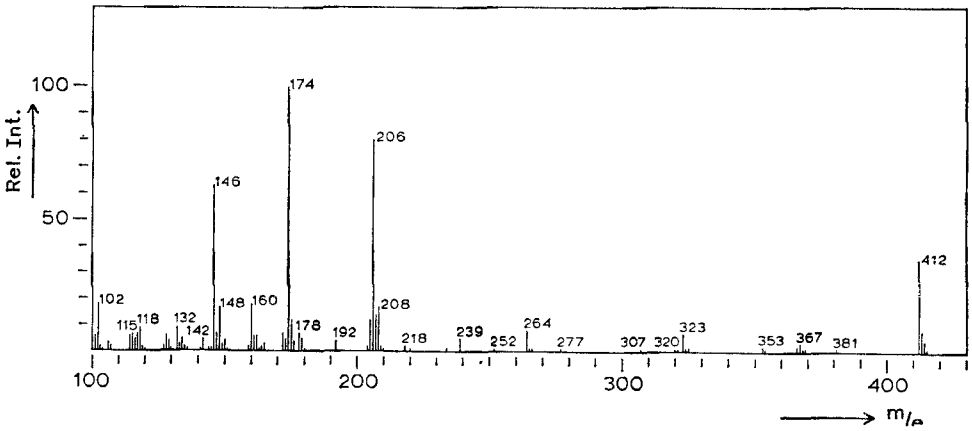
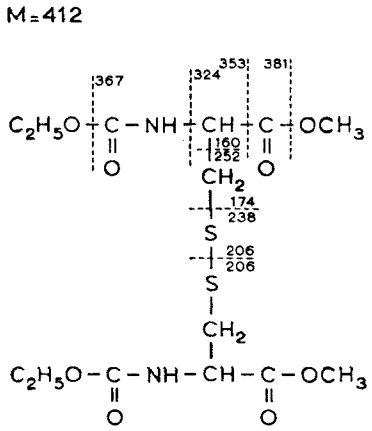


FIG. 4. Mass spectrum of bisethoxycarbonylcystine bismethyl ester. Only *m/e* values >100 are given.



SCHEME 4. Structure of bisethoxycarbonylcystine bismethyl ester, and its fragmentation pattern.

an improvement in the volatility of the derivatives and in the stabilisation of C-terminal fragments.

The ethoxycarbonyl-peptide methyl esters have a high volatility, as is apparent from the temperature of the ion source necessary to obtain a good spectrum. Recently Kiryushkin mentioned the relatively high volatility of ethoxycarbonylglycylleucylvaline methyl ester.[14] In Table 9 the volatility of several acylpeptide methyl esters is compared.

A further advantage of the ethoxycarbonyl derivatives is the relatively high abundance of the molecular peak and of the amino-acid sequence peaks in comparison to those of other acylpeptide methyl esters. In Table 10 the relative intensities of the sequence peaks and of the molecular peak of some valyltyrosylproline derivatives are expressed in % of Σ_{40}, calculated from S_0 (compare Prox[8]).

TABLE 4a. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF BISETHOXYCARBONYLCYSTINE BISMETHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
102	102·0013	102·0014	C_3H_4NOS	$206 - OCH_3 - COOC_2H_5$
102	**102·0377**	**102·0377**	C_4H_8NS	$206 - COOCH_3 - CO_2 - H$
102	102·0554	102·0555	$C_4H_8NO_2$	$C_2H_5O - CO - \overset{\oplus}{N}H{=}CH_2$
118	117·9958	117·9963	$C_3H_4NO_2S$	$206 - COOCH_3 - C_2H_5$
118	118·0086	118·0088	$C_4H_6O_2S$	$206 - NHCOOC_2H_5$
128	**127·9804**	**127·9806**	$C_4H_2NO_2S$	$206 - OCH_3 - OC_2H_5 - 2H$
128	128·0342	128·0348	$C_5H_6NO_3$	$367 - 238 - H$
132	132·0120	132·0119	$C_4H_6NO_2S$	$206 - COOC_2H_5 - H$
132	132·0286	132·0297	$C_4H_6NO_4$	$160 - C_2H_5 + H$
134	133·9733	133·9734	$C_3H_4NOS_2$	$238 - OCH_3 - COOC_2H_5$
134	134·0082	134·0077	$C_4H_6NO_2{}^{34}S$	sulfur isotope peak of 132
134	134·0276	134·0276	$C_4H_8NO_2S$	$206 - COOC_2H_5 + H$
174	174·0222	174·0225	$C_6H_8NO_3S$	$206 - OCH_3 - H$
174	174·0761	174·0766	$C_7H_{12}NO_4$	see structure
206	**206·0491**	**206·0487**	$C_7H_{12}NO_4S$	see structure
208	208·0447	208·0445	$C_7H_{12}NO_4{}^{34}S$	sulfur isotope peak of 206
208	208·0644	208·0644	$C_7H_{14}NO_4S$	$206 + 2H$
412	412·0963	412·0974	$C_{14}H_{24}N_2O_8S_2$	see structure

TABLE 4b. INTERPRETATION OF METASTABLE PEAKS IN THE MASS SPECTRUM OF BISETHOXYCARBONYLCYSTINE BISMETHYL ESTER

*m**	$m_1^+ \rightarrow m_2^+$	Eliminated group
253·2	412 → 323	$NH_2COOC_2H_5$
197·4	353 → 264	$NH_2COOC_2H_5$
147·0	**206 → 174**	**S**
131·4	323 → 206	$S(CH)_2COOCH_3$
126·7	239 → 174	HS_2
122·5	174 → 146	CO
115·9	174 → 142	CH_3OH

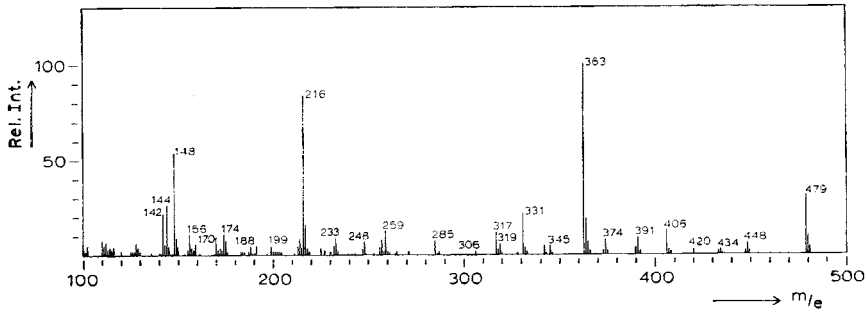
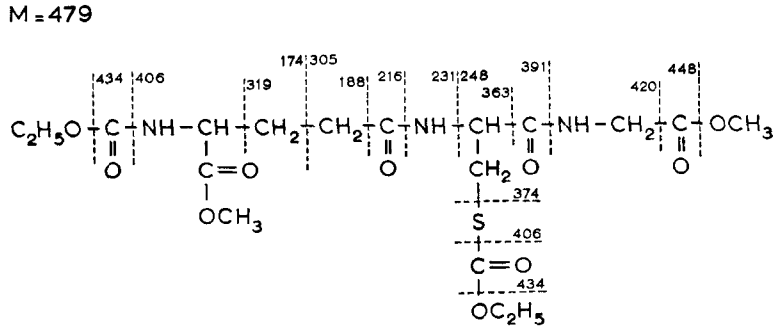


FIG. 5. Mass spectrum of ethoxycarbonyl-γ-glutamyl(methyl ester)-(S-ethoxycarbonyl)cysteinylglycine methyl ester. Only *m/e* values >100 are given.



SCHEME 5. Structure of ethoxycarbonyl-γ-glutamyl(methyl ester)-(S-ethoxycarbonyl)-cysteinylglycine methyl ester and its fragmentation pattern.

TABLE 5. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF ETHOXYCARBONYL-γ-GLUTAMYL(METHYL ESTER)-(S-ETHOXYCARBONYL)CYSTEINYLGLYCINE METHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
159	159·0114	159·0116	$C_6H_7O_3S$	391 − 231 − H
159	159·0227	159·0228	$C_5H_7N_2O_2S$	305 − $COOC_2H_5$ − CH_2COOCH_3
159	159·0764	159·0770	$C_6H_{11}N_2O_3$	231 − $COOC_2H_5$ + H
170	170·0813	170·0817	$C_8H_{12}NO_3$	363 − $NHCOOC_2H_5$ − $SCOOC_2H_5$
257	257·0584	257·0596	$C_{10}H_{13}N_2O_4S$	420 − $NHCOOC_2H_5$ − $COOC_2H_5$ − 2H
257	257·1119	257·1137	$C_{11}H_{17}N_2O_5$	363 − $SCOOC_2H_5$ − H
259	259·1278	259·1294	$C_{11}H_{19}N_2O_5$	363 − $SCOOC_2H_5$ + H
342	342·1307	342·1301	$C_{14}H_{20}N_3O_7$	374 − OCH_3 − H
479	479·1565	479·1574	$C_{18}H_{29}N_3O_{10}S$	see structure

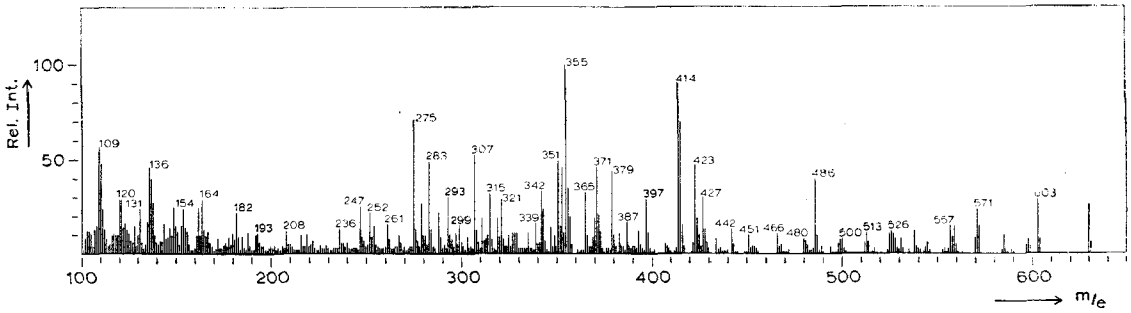
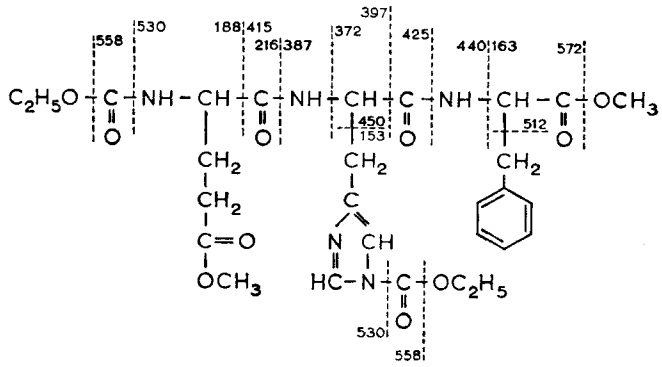


FIG. 6. Mass spectrum of ethoxycarbonyl-α-glutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidylphenylalanine methyl ester. Only *m/e* values >100 are given.

M = 603



SCHEME 6. Structure of ethoxycarbonyl-α-glutamyl(methyl ester)-(im-N-ethoxycarbonyl)histidylphenylalanine methyl ester, and its fragmentation pattern.

TABLE 6. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF ETHOXYCARBONYL-α-GLUTAMYL(METHYL ESTER)-(IM-N-ETHOXYCARBONYL)HISTIDYLPHENYLALANINE METHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
164	164·0454	164·0460	$C_7H_6N_3O_2$	425 − 188 − $COOC_2H_5$
164	164·0825	164·0824	$C_8H_{10}N_3O$	397 − $NHCOOC_2H_5$ − CH_2COOCH_3 − $COOC_2H_5$ + H
275*	275·0783	275·0780	$C_{12}H_{11}N_4O_4$	$C_{11}H_{12}N_4O_3$ + CO − H
275	275·1136	275·1117	$C_{10}H_{17}N_3O_6$	450 − $CH_2C_6H_5$ − $CH_2CH_2COOCH_3$ + 3H
293	293·1228	293·1250	$C_{13}H_{17}N_4O_4$	425 − $COOCH_3$ − $COOC_2H_5$
434	434·1324	434·1311	$C_{18}H_{20}N_5O_8$	512 − OC_2H_5 − OCH_3 − 2H
538*	538·1788	538·1785	$C_{22}H_{28}N_5O_{11}$	M′ − $CH_2C_6H_5$ − H
559	559·2601	559·2642	$C_{27}H_{37}N_5O_8$	603 − CO_2
603	603·2515	603·2540	$C_{28}H_{37}N_5O_{10}$	see structure
630*	630·2366	630·2411	$C_{29}H_{36}N_5O_{11}$	M′ = (M + 27)

* These peaks are derived from the product which gives the peak with the composition $C_{29}H_{36}N_5O_{11}$ i.e. (M + 27).

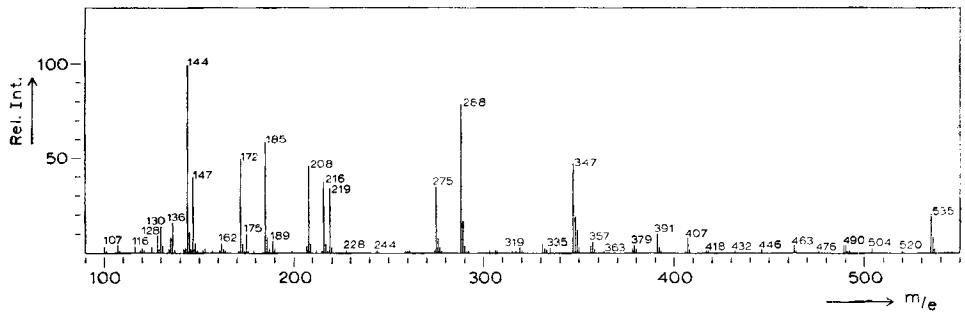
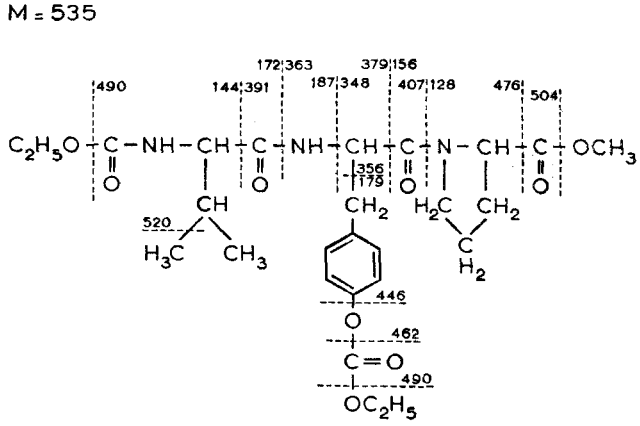


FIG. 7. Mass spectrum of ethoxycarbonylvalyl-(O-ethoxycarbonyl)tyrosylproline methyl ester. Only *m/e* values >100 are given.



SCHEME 7. Structure of ethoxycarbonylvalyl-(O-ethoxycarbonyl)tyrosylproline methyl ester, and its fragmentation pattern.

TABLE 7a. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF ETHOXYCARBONYLVALYL-(O-ETHOXYCARBONYL)TYROSYLPROLINE METHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
116	116·0709	116·0711	$C_5H_{10}NO_2$	144 − C_2H_5 + H
535	535·2518	535·2530	$C_{26}H_{37}N_3O_9$	see structure

TABLE 7b. INTERPRETATION OF METASTABLE PEAKS IN THE MASS SPECTRUM OF ETHOXYCARBONYLVALYL-(O-ETHOXYCARBONYL)TYROSYLPROLINE METHYL ESTER

*m**	$m_1^+ \rightarrow m_2^+$	Eliminated group
309·6	535 → 407	
280·2	391 → 331	$HCOOCH_3$
239·0	347 → 288	$COOCH_3$
225·1	535 → 347	$NH_2COCH[CH(CH_3)_2]NHCOOC_2H_5$
169·7	275 → 216	$COOCH_3$
166·5	288 → 219	
156·5	189 → 172	NH_3
120·6	172 → 144	CO

Besides these definite advantages of the application of the ethoxycarbonylpeptide derivatives for mass spectrometric analyses, there is in some cases the complication of a peak at M′ = [M + 26] or [M + 27] and a few peaks corresponding with this peak M′ (M′ minus OCH_3, M′ minus OC_2H_5). This peak probably originates from the introduction of one more ethoxycarbonyl group than could be expected, followed by the elimination of ethanol. These peaks were recorded for the valyltyrosylproline, valyllysylvalyltyrosylproline and glutamylhistidylphenylalanine derivatives. This feature is under investigation.

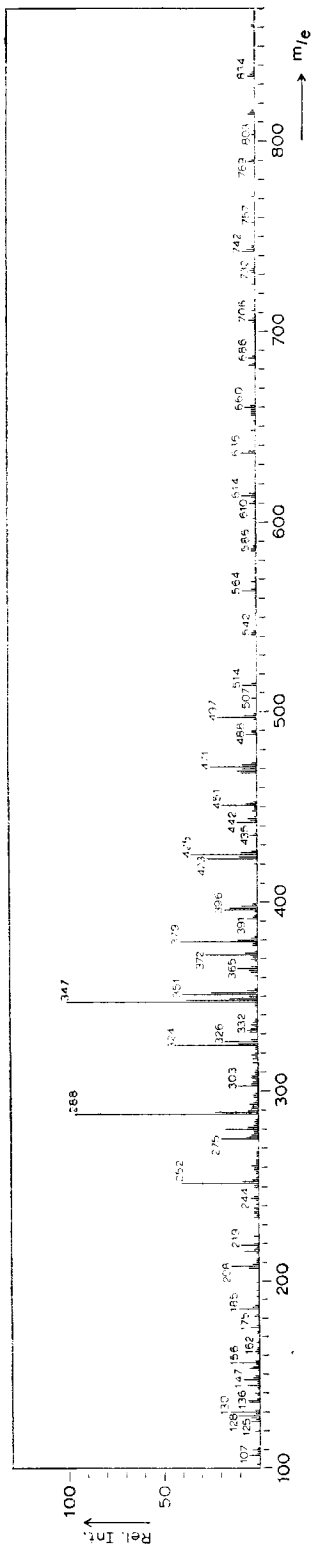
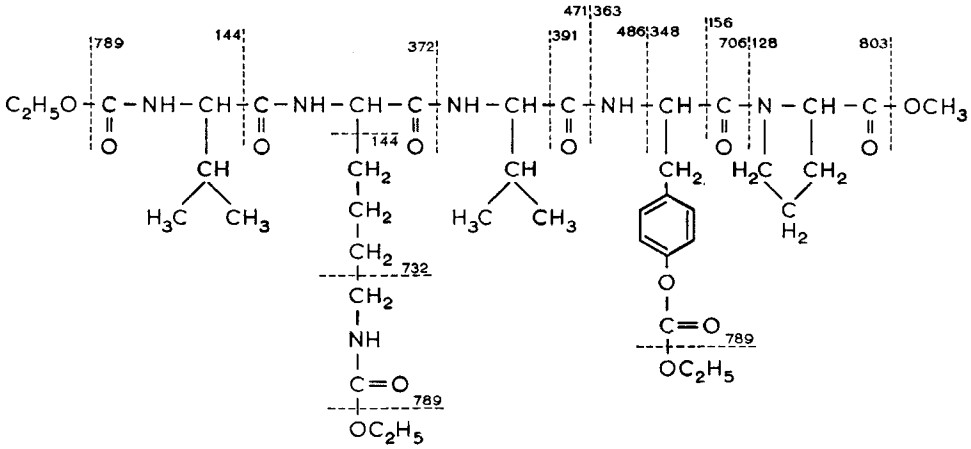


FIG. 8. Mass spectrum of ethoxycarbonylvalyl-(N-ethoxycarbonyl)lysylvalyl-(O-ethoxycarbonyl)tyrosylproline methyl ester.
Only *m/e* values >100 are given.

M = 834



SCHEME 8. Structure of ethoxycarbonylvalyl-(N-ethoxycarbonyl)lysylvalyl-(O-ethoxycarbonyl)tyrosylproline methyl ester, and its fragmentation pattern.

TABLE 8. DETERMINED AND CALCULATED *m/e* VALUES OF SOME PEAKS OF THE MASS SPECTRUM OF ETHOXYCARBONYLVALYL-(N-ETHOXYCARBONYL)LYSYLVALYL-(O-ETHOXYCARBONYL)-TYROSYLPROLINE METHYL ESTER

m/e	Measured value	Calculated value	Empirical formula	Fragment
130	130·0873	130·0868	$C_8H_{12}NO_2$	128 + 2H
185	185·0924	185·0926	$C_8H_{13}N_2O_3$	363 − $CH_2C_6H_4OCOOC_2H_5$ + 1H
216	216·1032	216·1024	$C_{13}H_{14}NO_2$	348 − $COOC_2H_5$ − $COOCH_3$
252	252·1324	252·1348	$C_{12}H_{18}N_3O_3$	372 − $COOC_2H_5$ − OC_2H_5 − 2H
324	324·1545	324·1559	$C_{15}H_{22}N_3O_5$	834 − $CH_2C_6H_4OCOOC_2H_5$ − $(CH_2)_4NHCOOC_2H_5$ − $NHCOCH[CH(CH_3)_2]$-$NHCOOC_2H_5$
365	365·1735	365·1712	$C_{18}H_{25}N_2O_6$	363 + 2H
442	442·2665	442·2665	$C_{20}H_{36}N_5O_6$	486 − OC_2H_5 + H
788	788·3953	788·3956	$C_{38}H_{56}N_6O_{12}$	789 − H
789	789·3996	789·4034	$C_{38}H_{57}N_6O_{12}$	see structure
803	803·4161	803·4191	$C_{39}H_{59}N_6O_{12}$	see structure
814*	814·3697	814·3749	$C_{39}H_{54}N_6O_{13}$	M′ − OC_2H_5 − H
834	834·4373	834·4375	$C_{40}H_{62}N_6O_{13}$	see structure
860*	860·4185	860·4167	$C_{41}H_{60}N_6O_{14}$	M′ = [M + 26]

* These peaks are derived from the product with molecular-formula $C_{41}H_{60}N_6O_{14}$ i.e. [M + 26].

TABLE 9. COMPARISON OF THE VOLATILITY OF A FEW ACYLPEPTIDE METHYL ESTERS

Derivative*	Ion source temperature
DNP-val-(DNP)tyr-pro-OCH_3[3]	220°
Capr-val-(Capr)tyr-pro-OCH_3[4]	190°
Bz-val-(Bz)tyr-pro-OCH_3	190°
Ec-val-(Ec)tyr-pro-OCH_3	140°
DNP-val-(DNP)lys-val-(DNP)tyr-pro-OCH_3	even at 300° too low volatility
Capr-val-(Capr)lys-val-(Capr)tyr-pro-OCH_3[4]	280°
Ec-val-(Ec)lys-val-(Ec)tyr-pro-OCH_3	210°
Z-trp-gly-OC_2H_5[10]	200°
Ec-trp-gly-OCH_3	120°

List of abbreviations
DNP = 2,4-dinitrophenyl
Capr = caproyl
Bz = benzoyl
Z = benzyloxycarbonyl
Ec = ethoxycarbonyl

TABLE 10

Protecting group	Acyl—NH—CH(R)—C(=O)—NH—CH(R′)—C(=O)—NH—CH(R″)—C(=O)—OCH_3								$\sum_{S_0}^{S_6}$	$\sum_{S_1}^{S_6}$
	S_0	S_1	S_2	S_3	S_4	S_5	S_6	$M^{\oplus}$		
DNP	2·08	2·20	—	—	—	—	0·12	0·87	4·40	2·32
Caproyl	1·13	3·08	4·00	0·21	1·74	—	0·21	2·46	10·37	9·24
Benzoyl	3·67	6·34	7·35	—	0·11	—	0·09	0·12	17·56	13·89
Ethoxycarbonyl	0·64	12·85	6·43	0·39	1·03	0·13	0·26	2·70	21·73	21·09

EXPERIMENTAL

Diethylpyrocarbonate was prepared from ethylchlorocarbonate according to Boehm and Metha[15].

Diazomethane was prepared from N-[tolylsulfonyl-(4)]-N-methylnitrosamide according to Backer and de Boer[16].

Preparation of ethoxycarbonyl derivatives of peptide methyl esters. 0·01 mmol of a peptide was dissolved in 2 ml H_2O, subsequently 0·015 mmol $NaHCO_3$/COOH group and 0·01 mmol diethylpyrocarbonate/acylable group were added. The mixture was shaken for ½ to 1 hour at room temperature, then acidified with 1 N HCl and extracted with 2 ml ethylacetate.

(a) The ethylacetate layer was washed twice with 1 ml H_2O. Diazomethane dissolved in diethylether was added to the ethylacetate solution of the ethoxycarbonyl-peptide until the solution remained pale yellow. After 15 minutes the excess of diazomethane was removed by evaporation.

(b) The ethoxycarbonyl derivative of the peptide gly-ser was more soluble in H_2O than in ethylacetate. In this case the water-layer was lyophylised. The residue was dissolved in 2 ml methanol and esterified with diazomethane as described above.

Besides the amino group, the imidazole ring of histidine, the sulfhydryl group of cysteine and the phenolic hydroxyl group of tyrosine were also ethoxycarbonylated (compare Mühlrad[17]). The peptide derivatives were purified by thin-layer chromatography on Kiesel gel G (Merck) using the solvent system petroleum ether (boiling range 40° to 60°C):diethylether = 15:20. For the localisation of the spots a parallel chromatogram was developed with Cl_2/*o*-tolidine-KI[18]. The peptide derivatives were eluted with ethylacetate.

The 70 eV mass spectra were recorded with an MS-9 mass spectrometer (AEI) at an ion chamber temperature of 75° to 210°C.

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