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Characterization of N-ethoxycarbonyl ethyl esters of amino acids by mass spectrometry

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ABSTRACT

Derivatization of amino acids by using ethyl chloroformate-ethanol-pyridine provides volatile products, N-ethoxycarbonyl amino acid ethyl esters (ECEEs), which are easily amenable to GC or GC-MS analysis. MS behavior of these compounds under electron-impact has been studied. The fragments observed in the spectra facilitate recognition of commonly occurring protein amino acids and characterization of unknown analogues.

INTRODUCTION

Analysis of protein hydrolysates for the usual amino acids by GC is now routine (for review articles, see refs. l-3). The foundation for the most commonly used method was laid by Gehrke et al. [4], who developed the procedure for quantitative derivatization to provide N(O,S)-trifluoroacetyl (TFA) amino acid n-butyl esters (TAB amino acids). Other variants include some closely related derivatives of analogous perfluoroacyl alkyl esters [1,2].

Procedures for preparation of TAB and related derivatives require two reactions. Although much progress has been made in this respect, a procedure suitable for the quantitative and reproducible derivatization of all protein amino acids in a single reaction remains to be developed. A modified procedure using a mixture of pentafluoropropionic anhydride (PFPA) and hexafluoroisopropanol (HFIP) was introduced to provide N-PFPA amino acid HFIP esters in one step [5,6]. Because these derivatives, contrary to what may be expected, do not form stable anions for all amino acids, their application was mainly restricted to the analysis of certain aromatic amino acids in body fluids. A number of new derivatization methods have been reported in the past decade. These methods include silylation, especially *tert*.-butyldimethylsilylation (TBDMS) [7], an improved procedure superseding the previously employed trimethylsilylation, and the formation of cyclic oxazolidinone derivatives by condensation with 1,3-dichlorotetrafluoroacetone followed by treatment with pentafluoropropionic anhydride [8]. The TBDMS method shows promise in the analysis of protein amino acids, including asparagine, glutamine, and arginine; however, the interpretation of the mass spectra

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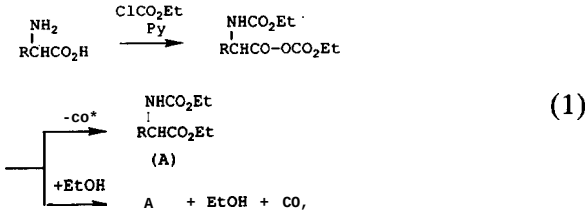
of TBDMS derivatives is somewhat more ambiguous than is the interpretation of the mass spectra of N-TFA amino acid alkyl esters. Relatively few non-protein amino acids have been analyzed this way. The oxazolidinone procedure [8], though simple in operation, requires two chromatographic columns for the resolution of all proteic amino acids.

A completely new approach has been reported recently by Hušek and Sweeley [9–11] involving simultaneous N(O,S)-derivatization with ethyl chloroformate (ECF) in water-ethanol-pyridine (Py) (reaction 1). As depicted in Fig. 1, the derivatives, N-ethoxycarbonyl amino acid ethyl esters (ECEE), show excellent resolution on a capillary column and the analysis, including the derivatization is completed within 10 min.



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This paper reports a comprehensive study on the mass spectrometric fragmentation of this interesting family of new derivatives. Interpretation of these spectra may facilitate recognition of individual amino acids and pave the way for the structure elucidation of modified amino acids.

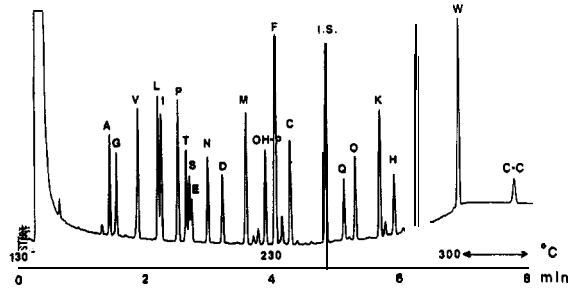


Fig. 1. Gas chromatogram of 22 ECEE amino acids. GC conditions: DB-1701 column, 1 μm, 30 m × 0.53 mm I.D., programmed at 25°C/min between 130 and 300°C, 70 kPa hydrogen. Internal standard (IS.): p-chlorophenylalanine.

EXPERIMENTAL

The amino acids and ethyl chloroformate were purchased from Sigma (St. Louis, MO, USA). The ECEE derivatives were prepared according to the procedure reported previously [9–11]. Analysis by GC-MS was carried out on a JEOL AX-505H double-focusing mass spectrometer coupled to a Hewlett-Packard 5890J gas chromatograph. GC separation employed DB-1701 (30 m length × 0.53 mm I.D. fused-silica capillary column with a 1.0-μm film coating; or 15 m length × 0.25 mm I.D. × 0.25 μm film thickness) available from J. & W. Scientific (Rancho Cordova, CA, USA). Direct (splitless) injection was used. Helium gas flow was approximately 1 ml/min. MS conditions were as follows: interface temperature 280°C, ion source temperature cu. 150–200°C, electron energy was 70 eV, scan rate of the mass spectrometer was 1 s/scan over the m/z range 50-500.

RESULTS AND DISCUSSION

In general, the interpretation of the described electron-impact (EI) mass spectra and the assignment of various ions were based on the knowledge on the fragmentation behaviors of some other types of amino acid derivatives [3]. The assignment of molecular ions was experimentally confirmed using gas chromatography-chemical ionization (methane) mass spectrometry, GC–CI(CH_4)-MS, when the expected peak was very weak or absent. Table I lists the mass values of the characteristic peaks in the spectra of individual amino acid derivatives.

Aliphatic amino acids

Fig. 2 shows the mass spectrum of the valine ECEE derivative as a representative of this group (glycine, alanine, valine, leucine, and isoleucine). As shown in Scheme 1, two fragmentation routes (a and b) are possible through the rupture of a carbon-carbon bond α to the amine group. Generally, path b is preferred over a because $^{\cdot}CO_2Et$, the fragment having higher ionization energy compared to that of the alkyl chain, is favored energetically to retain the unpaired electron and to become the neutral prod-

TABLE I

CHARACTERISTIC ION PEAKS IN EI SPECTRA OF ECEE DERIVATIVES OF AMINO ACIDS

Amino acid	M_r	Derivative [$M^{+\cdot}$]	Base peak m/z	M-73	M-145	Other important ions, m/z
Gly	75	175	102	102		
Ala	89	189	116	116		
Val	117	217	144	144	72	116
Leu	131	231	158	158	86	102
Ile	131	231	158	158	86	102
Pro	115	215	142	142	70	98
HPr	131	231	158	158	86	68
Ser	105	(205)	132	132	60	175, 129, 101, 86
Thr	119	(219)	129	146	74	175,101
AHBA[b]	119	219	103	146	74	117, 84
		155[d]	128			110, 83
DABA[c]	118	290	155	217		175, 129, 128, 115, 102, 83, 56
		172[d]	83	99		115, 83, 70, 56
Om	132	304	142			258, 70
		186[d]	141	113		129, 97, 70
Lys	146	318	156			272, 226, 84
Cys	121	293	220	220	148	174, 114, 102, 74
Cys^2	240	440	188	367		220, 174, 102
Met	149	249	175	176		188, 142, 129, 101, 61
Asp	133	261	188	188	116	142, 74, 70
Glu	147	275	202	202		156, 128, 84
p-Glu	129	157	84	84		
Asn	132	214	141	141	69	174,102
Gln	146	246	84	173		128
Phe	165	265	176	192	120	131, 102, 91, 74
Tyr	181	353	107	280	208	264,192
Trp	204	304	130	231		215
His	155	327	238	254	182	154, 81

[a] Mass value given in parentheses indicates the absence of a molecular ion peak in the spectrum.
[b] AHBA = γ-Amino-β-hydroxybutyric acid.
[c] DABA = 2,4-Diaminobutyric acid.
[d] Minor derivatixation product.

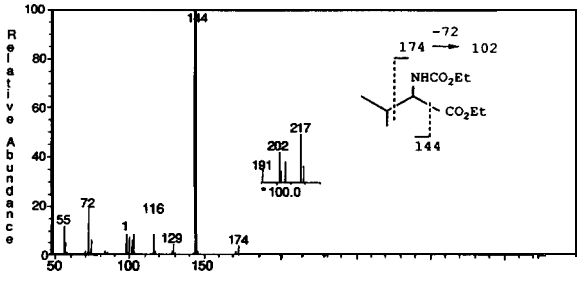


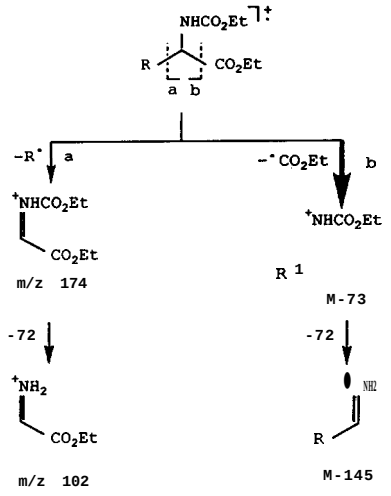
Fig. 2. Mass spectrum of ECEE valine (M, 217). The asterisk in the inset of the figures throughout the text denotes a multiplication factor.

uct. Subsequent fragmentation of the even-electron ions (EE^+) thus formed is dominated by the loss of 72 u ('CO_2Et − H'), giving rise to ions m/z 102 and [M −145], respectively.

Cyclic amino acids

The mass spectrum of the ECEE derivative of hydroxyproline is shown in Fig. 3 with the corresponding fragmentation pattern depicted in Scheme 2.

Similar to the pattern obtained from the aforementioned simple aliphatic derivatives, the



Scheme 1.

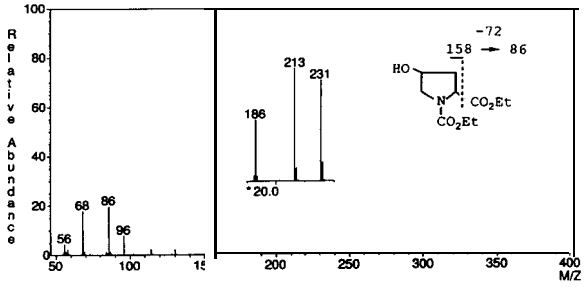
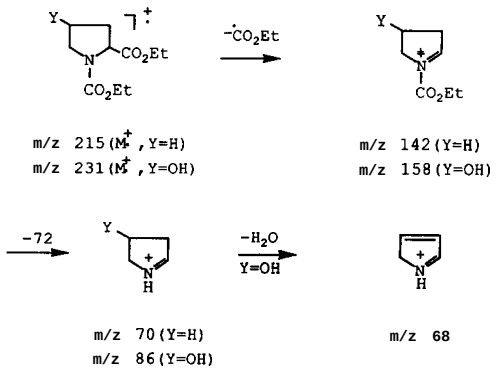


Fig. 3. Mass spectrum of ECEE 4-hydroxyproline (M_r 231).



Scheme 2.

highest peak in this spectrum represents the ion formed by a reaction sequence that originates in the splitting of the ester group to yield an ion at m/z 158 (m/z 142 for Pro). This ion loses ($^{\bullet}CO_2Et - H'$) to produce the characteristic even mass ion at m/z 86 (m/z 70 for Pro). Subsequent loss of H_2O from the latter ion results in the formation of an ion of m/z 68.

Another example of the cyclic amino acid series is the ECEE of pyroglutamic acid (Fig. 14), which will be discussed later in a section regarding acidic amino acids.

Hydroxy amino acids

In Fig. 4 the mass spectrum of the serine derivative is given, together with the fragmentation pathways shown in Scheme 3.

As discussed before, the process $[M]^+ \rightarrow [M - 73]^+ \rightarrow [M - 73 - 72]^+$ (*m/z* 132 and 60 in Fig. 4) remains prominent in the fragmentation of the hydroxy analogues. Two additional fragment peaks, m/z 102 and 86, are formed via rearrangements with concurrent removal of RCHO (R = H for Ser) and EtOH, respectively.

Another sequence of degradation is due to a McLafferty-type rearrangement, which is triggered by a hydrogen migration originating from the β-HO group to produce an abundant ion peak at m/z 175. A subsequent cyclization reaction leads to ions m/z 129 and 101. This sequence is a common feature for all amino acid derivatives having a labile hydrogen atom to permit transition via a six-membered ring.

The ECEE derivative of threonine behaves in a similar way, showing a mass shift of 14 u for ions that retain the side-chain $CH_3CH(OH)$- in the fragment.

Depicted in Fig. 5 is the mass spectrum obtained from a non-protein amino acid, γ-amino-β-hydroxybutyric acid (AHBA), which gives an open-chain derivative with $M^{+\bullet}$ at m/z 219.

Generally, the loss of an ester group is not a

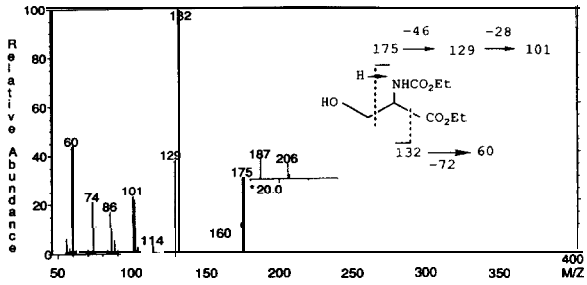
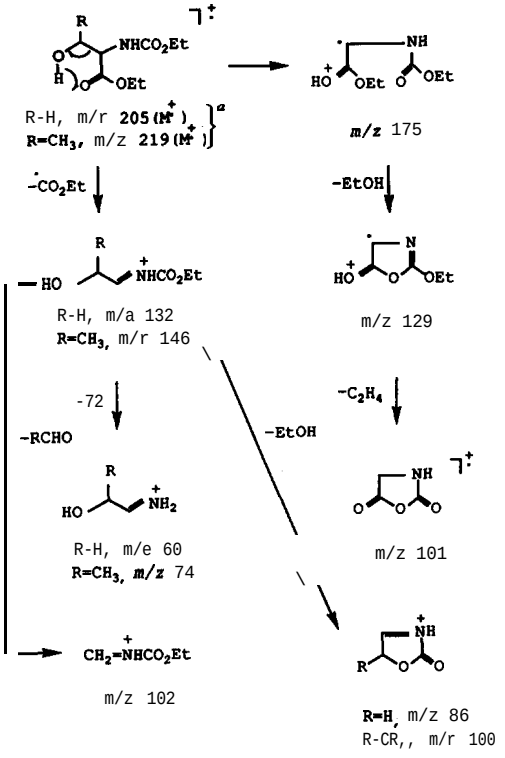


Fig. 4. Mass spectrum of ECEE serine (M_r 205). Fragments arise from $M^{+\bullet}$, although MH' (m/z 206) is observed.



Scheme 3. [a] **Derivatives not observed.**

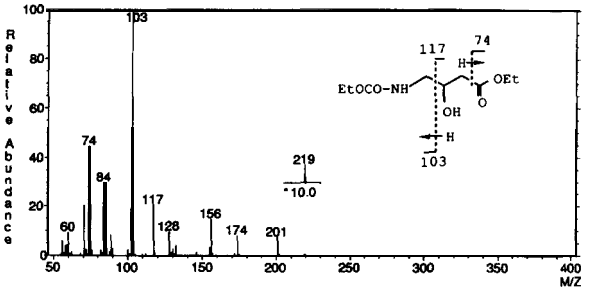
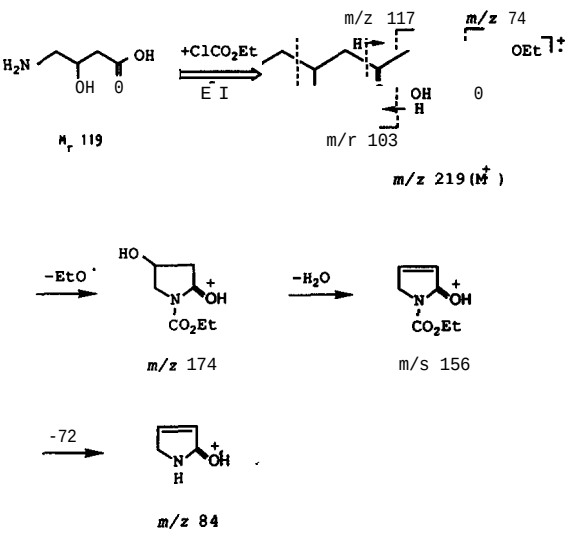


Fig. 5. Mass spectrum of ECEE γ-amino-β-hydroxybutyric acid (M_r 219).

significant process for y-amino acid derivatives [3]. As indicated in Scheme 4, there are four ions (m/z 74, 88, 103 and 117) formed by cleavage along the carbon chain, whereas the terminal immonium ion ***m/z*** 103, resulting from a rearrangement involving hydrogen-transfer, is the base peak in the spectrum.

Another fragmentation process involves the loss of an **EtO•** radical from the molecular ion, resulting in the formation of a cyclic ion at ***m/z***



Scheme 4.

174 and its subsequent degradation product ions at ***m/z*** 156 and 84 (Scheme 4).

Like other y-amino acids, AHBA undergoes cyclization readily on treatment with ECF-Py to yield N-ethoxycarbonyl dehydropyrrolidone ($M^{+\bullet}$ at 155) as a minor reaction product. Subsequent double hydrogen rearrangement (**"McLafferty** + 1" rearrangement) is likely responsible for the formation of m/z 128 and daughter ions ***m/z*** 110 and 83 therefrom (Fig. 6; Scheme 5).

Sulfur-containing amino acids

Figs. 7 and 8 present the mass spectra of ECEE cysteine and methionine, respectively.

These amino acids have the same typical fragments as those obtained from aliphatic amino

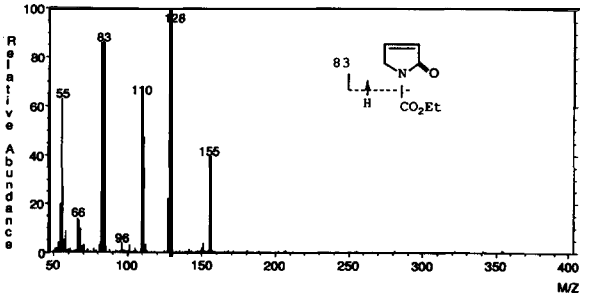
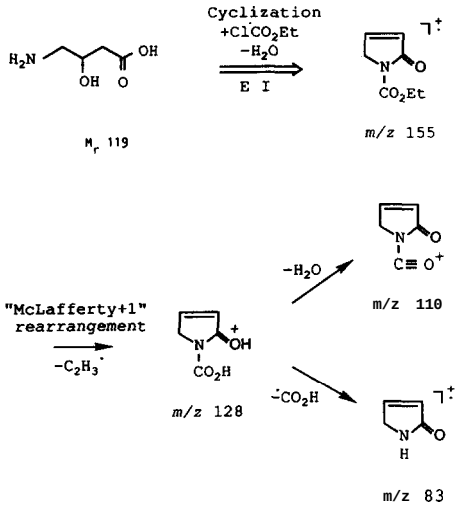


Fig. 6. Mass spectrum of the minor derivatization product of γ-amino-β-hydroxybutyric acid (M_r 155).



Scheme 5.

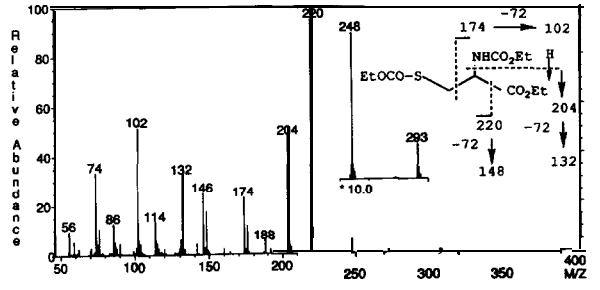


Fig. 7. Mass spectrum of ECEE cysteine (M_r 293).

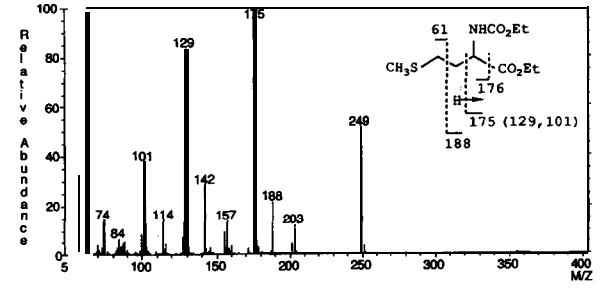
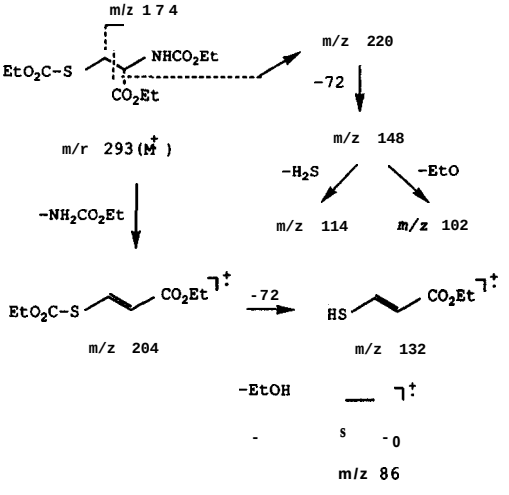


Fig. 8. Mass spectrum of ECEE methionine (M, 249).

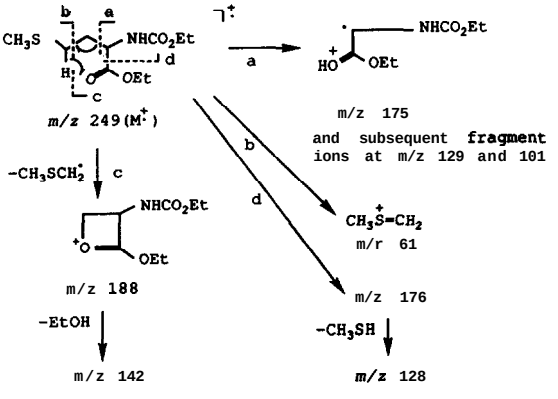
acids. Additional fragments were observed due to the presence of the sulfur atom. Thus, in the spectrum of the cysteine derivative (Fig. 7), a resonance-stabilized ion m/z 204 ($M_r - NH_2CO_2Et$) is formed through the McLafferty rearrangement with charge retention on the fragment that contains sulfur. The consecutive loss of ($^{\bullet}CO_2Et$ – H') and **EtOH** leads to another two sulfur-containing species m/z 132 and 86 (Scheme 6).

A more **prounced influence** exerted by the sulfur atom occurs in the spectrum of the methionine derivative (Fig. 8; Scheme 7). An intense ion peak m/z 175 (together with its product ions m/z 129 and 101) due to the McLafferty rearrangement is observed, which is initiated by the transfer of the labile hydrogen, that is activated by a-sulfur. Whereas the low mass end of the spectrum is dominated by the sulfonium ion $CH_3S{=}CH_2^+$ (m/z 61), an ion complementary to m/z 61 is displayed at m/z 188 [61 + 188 = 249 ($M^{+\bullet}$)].

The ECEE derivative of cystine (Fig. 9) is an



Scheme 6.



Scheme 7.

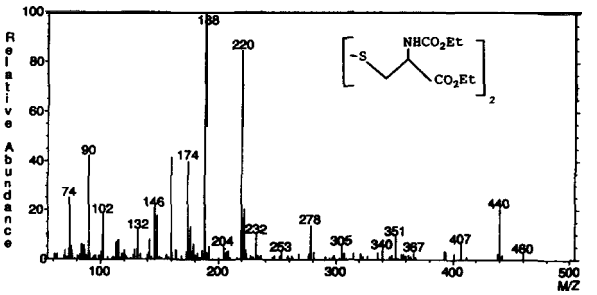


Fig. 9. Mass spectrum of ECEE cystine (M_r 440).

example where fragmentation occurs predominantly due to the influence of the sulfur atom. Two major peaks in the spectrum, ***m/z*** 220 and 188, are formed via homolytic fission of the -S-S- linkage and a-cleavage induced by the electropositive sulfur atom.

Basic amino acids

Because the basic amino acids [2,4-diaminobutyric acid (DABA), ornithine and lysine], after derivatization, demonstrate quite similar degradation patterns, their fragmentations are illustrated by the example of the ECEE derivative of ornithine (Fig. 10).

Generally, the loss of the ester function is not a significant process for these compounds because of the competing reactions due to the remote amino group. Typically, these ECEE amino acids undergo consecutive loss of $^{\bullet}CO_2Et$ and NH_2CO_2Et, which leads to the formation of the stable cyclic immonium ions m/z 128, 142 and 156 (for n = 2, 3 and 4), respectively. This synchronic process is rationalized by a S_{Ni} reaction mechanism (see, for example, ref. 12) as presented in Scheme 8.

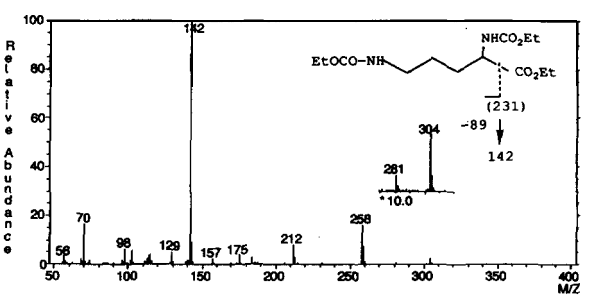
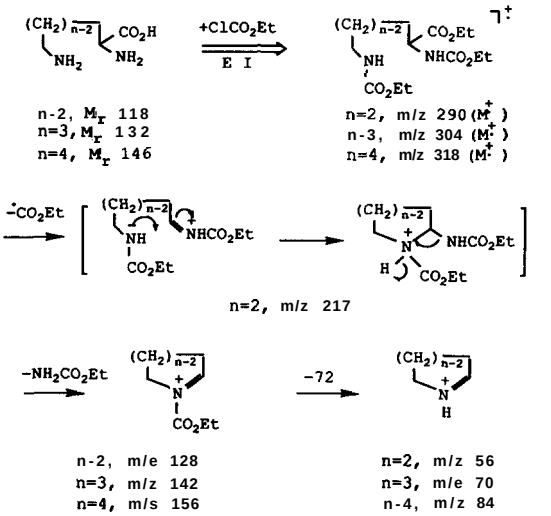


Fig. 10. Mass spectrum of ECEE omithine (M_r 304).

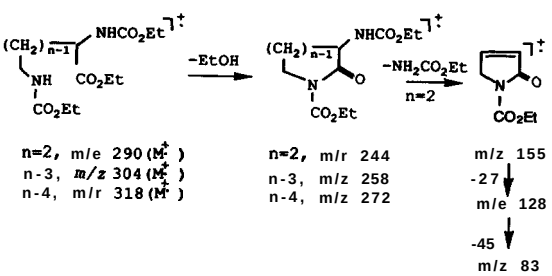


Scheme 8.

Another noteworthy feature of the spectrum depicted in Fig. 10 is the ejection of **EtOH** from the molecular ion with the formation of the cyclic ions ***m/z*** 244,258 and 272 (for n = 2, 3 and 4), respectively (Scheme 9).

Analogous to the methionine derivative, the ECEE **DABA** undergoes a **McLafferty** rearrangement to yield product ions at ***m/z*** 175, 129 and 101 (Table I).

Two minor by-product peaks are sometimes detected in the gas chromatogram for **DABA** and omithine (Fig. 11). These compounds show common peaks [M-45]+, [M-57]+ and $[M-73]^+$ by a mechanism that is not yet clear. The respective ions ***m/z 83*** and 97 are formed as a result of ejecting NH_2CO_2Et from each of the molecular ions (Scheme 10).



Scheme 9.

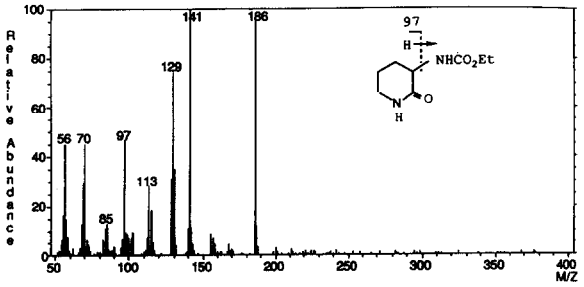
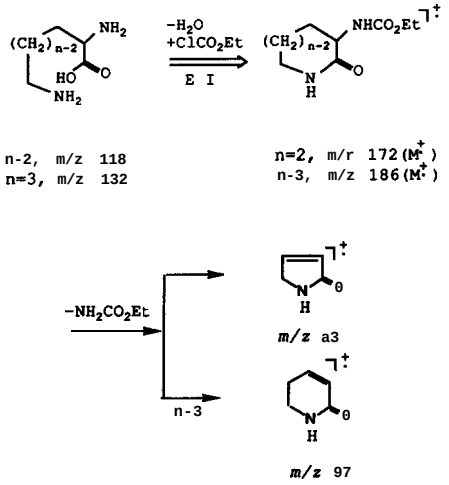


Fig. 11. Mass spectrum of the minor derivatization product of omithine (M_r 186).



Scheme 10.

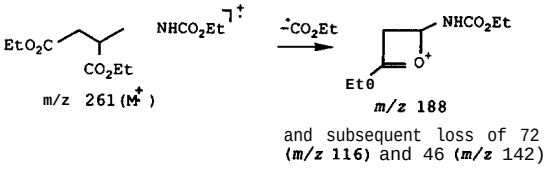
Acidic amino acids

The spectrum pattern of the aspartic acid derivative is simple and dominated by the ion at m/z 188 (M, $-{}^{\bullet}CO_2Et$) (Fig. 12). This pattern may be explained by the formation of a stabilized cyclic oxonium ion (m/z **188**), which **facilitates** the cleavage of the C-C bond connecting the ester group (Scheme 11).

Shown in Fig. 13 is the mass spectrum of the glutamic acid derivative. The loss of the **α-CO_2Et** initiates a sequence reaction leading to the pyrrolidinone ions at m/z 156 and 84 (Scheme 12).

Fig. 14 presents the mass spectrum of another derivatization product of glutamic acid, which is produced as a result of dehydration to form pyroglutamic acid followed by esterification by ECF. Because other a-cleavages do not lead to fragmentation, the mass spectrum is comprised almost totally of m/z 84, which is formed by splitting an ester radical from the molecular ion.

Fig. 15 presents the mass spectrum of the



Scheme 11.

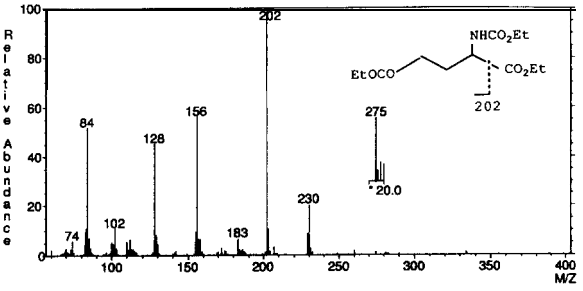


Fig. 13. Mass spectrum of ECEE glutamic acid (M_r 275).

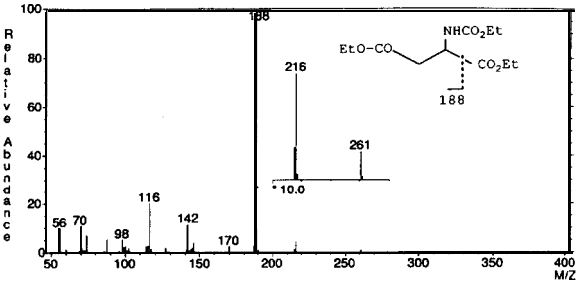
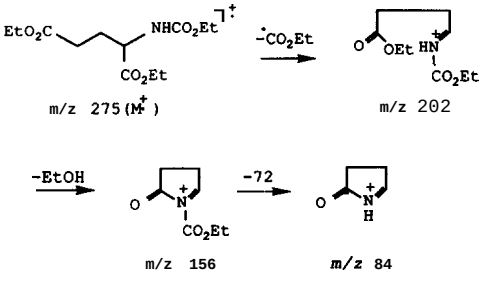


Fig. 12. Mass spectum of ECEE aspartic acid (M_r 261).



Scheme 12.

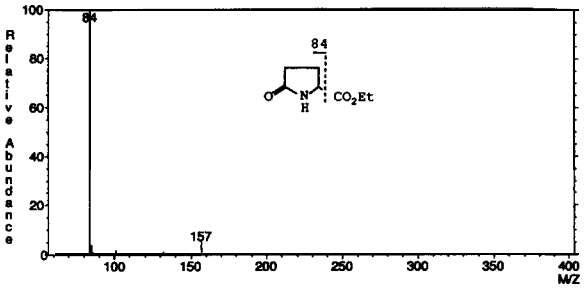


Fig. 14. Mass spectrum of pyroglutamic acid derivative (M_r 157).

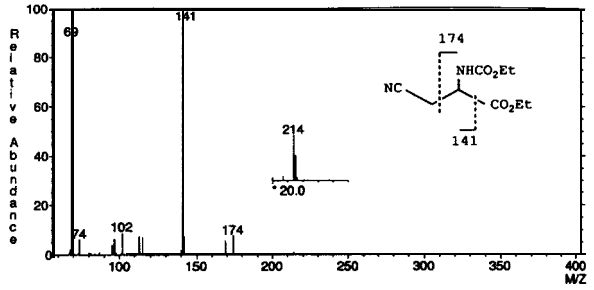
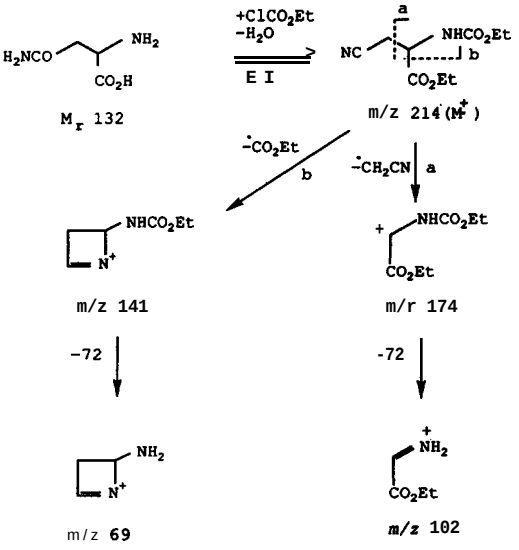


Fig. 15. Mass spectrum of asparagine derivative (M_r 214).

ECEE derivative of asparagine. Instead of the usual N-ethoxycarbonylation-C-esterification, a dehydration of the terminal carboxamide may occur to afford the corresponding **nitrile [$M^{+\bullet}$ =** m/z **214]** (Scheme 13). This pattern is analogous to some cases (for example, maleamic and phthalimic acids) documented in the literature [**13**]. This dehydration product undergoes further fragmentation according to sequences a (loss of **$^{\bullet}CH_2CN$**) or b (loss of **$^{\bullet}CO_2Et$**) to give product ions ***m/z*** 174 and 102, or ***m/z*** 141 and 69, respectively.



Scheme 13.

The fragmentation of glutamine, a homologue of asparagine with the 'CONH, group one more methylene from the carboxyl, takes place in a different way (Fig. 16, Scheme 14) where the prevailing event is the cyclization induced by functional group interaction between 'CONH, and the amino terminus in the **EE^+** species **(*m/z*** 173 = **$M_r - {}^{\bullet}CO_2Et$)**.

Aromatic and heterocyclic amino acids

In general, ECEE amino acids with an aromatic (Ar) or heterocyclic group in the side chain afford much more abundant ions due to loss of side chain **($ArCH_2{}^{\bullet}$)**. Fig. 17 presents the mass spectrum of the ECEE derivative of **phen**ylalanine. There are essentially two competing primary fragmentation reactions: elimination of

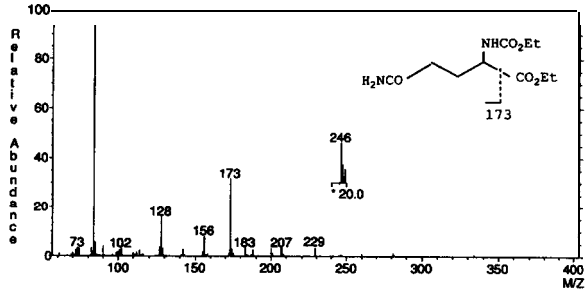
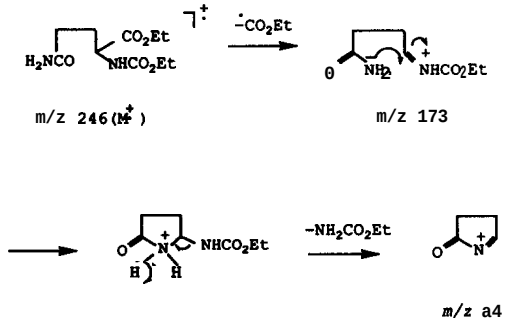


Fig. 16. Mass spectrum of ECEE glutamine (M_r 246).



Scheme 14.

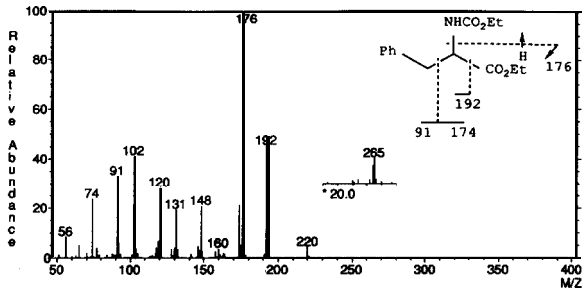
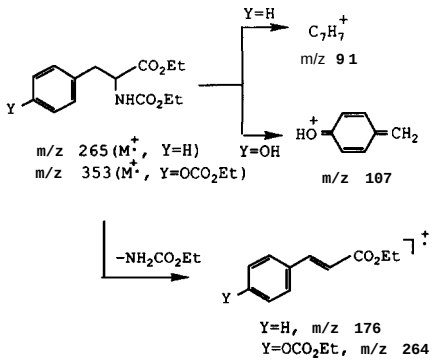


Fig. 17. Mass spectrum of ECEE phenylalanine (M_r 265).

NH_2CO_2Et from $M^{+\cdot}$ to give a conjugated ion *m/z* 176, and formation of a benzyl-type ion, $ArCH_2^+$ (m/z 91 in Fig. 17) (Scheme 15).

The mass spectra and interpretation for derivatives of tryptophan and histidine are given in Fig. 18 (Scheme 16) and Fig. 19 (Scheme 17), respectively. Different from other amino acid derivatives, ECEE histidine affords not $ArCH_2^+$, but $ArCH_3^{+\cdot}$ by a six-membered ring hydrogen transfer.



Scheme 15.

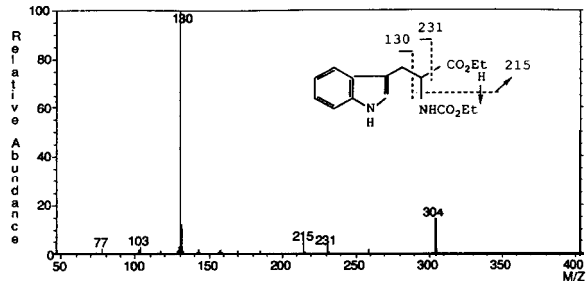
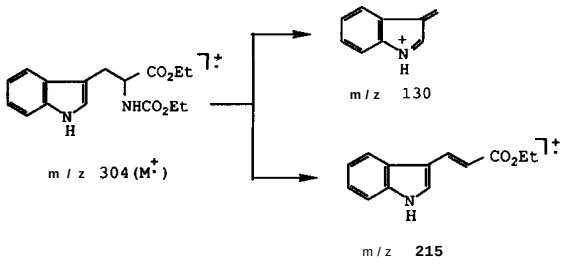


Fig. 18. Mass spectrum of ECEE tryptophan (M_r 304).



Scheme 16.

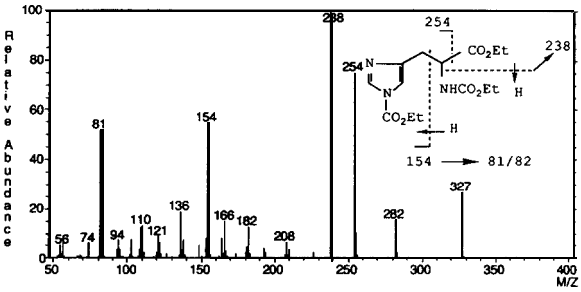
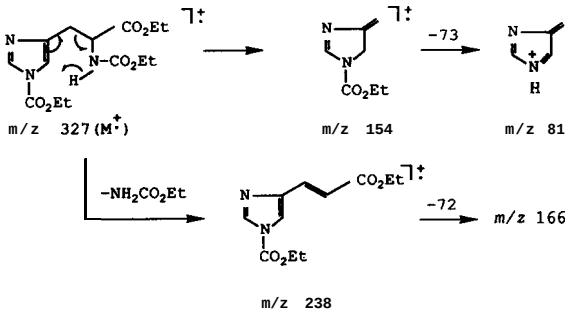


Fig. 19. Mass spectrum of ECEE histidine (M_r 327).



Scheme 17.

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