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GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF SOME BIOLOGICALLY IMPORTANT SHORT CHAIN ACID BUTYL ESTERS

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SUMMARY

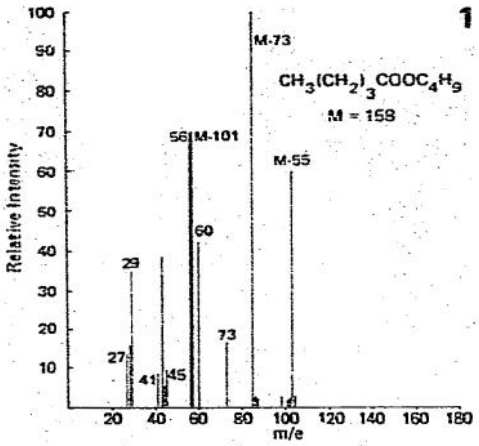
Mass spectra of *n*-butyl esters of selected biologically important short chain fatty acids were obtained by using the technique of gas–liquid chromatography–mass spectrometry. The results indicate that simple cleavage is responsible for the primary fragmentation of the molecules. The mass spectral data are considered to be advantageous for the identification of unknown short chain acids.

INTRODUCTION

In recent years, gas–liquid chromatography (GLC) has become an established technique for the determination of short chain acids in biological material[1]. In most instances, these acids are converted to ester derivatives before GLC analysis in order to increase their volatility and prevent their destruction (decarboxylation, dimerization), which may occur when they are analyzed in the free state[2,3]. Various ester derivatives have been reported[2–4]. However, we prefer *n*-butyl ester (*n*-BE) derivatives because they are easy to prepare and give excellent resolution on common GLC stationary phases[5].

Identification of components by GLC is often an arduous task. With biological materials, difficulties of identification are compounded because the unknown is frequently present in small amounts in an extract which contains a wide spectrum of metabolites. Moreover, fractionation, purification, and derivatization of the sample before GLC analysis may produce artifacts which, except for their structure, seem to bear a striking resemblance to the unknown. Although short chain acid esters from biological fluids are effectively separated by GLC, their identification by this technique is, at best, only tentative. Absolute identification is possible, however, by interfacing the gas chromatograph with a mass spectrometer and recording the mass spectra of each separated component as it elutes from the GLC column. This combined GLC–mass spectrometry (MS) technique has proved to be a powerful analytical tool in various biological applications[5].

The MS pattern of the *n*-BE of short chain fatty acids has been restricted only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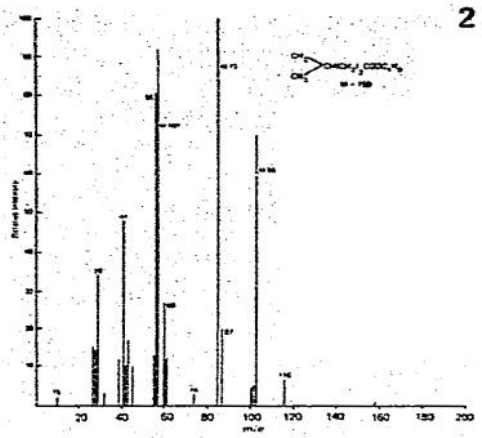
M-73
$CH_3(CH_2)_3COOC_4H_9$
M = 158
M-101
M-55
Relative Intensity
m/e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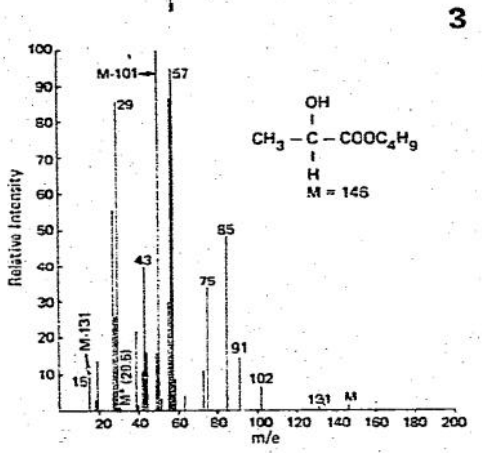
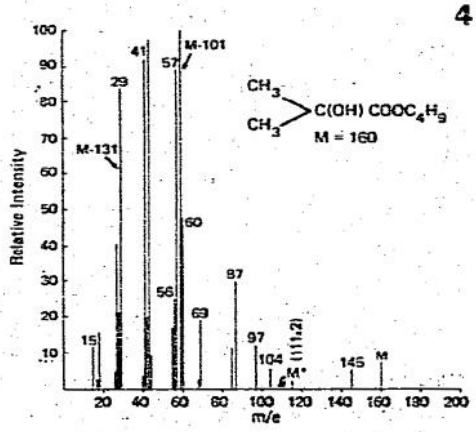
Relative Intensity
m/e


3
M-101
M-131
M+ (20.6)
$CH_3-C(OH)H-COOC_4H_9$
M = 146
Relative Intensity
m/e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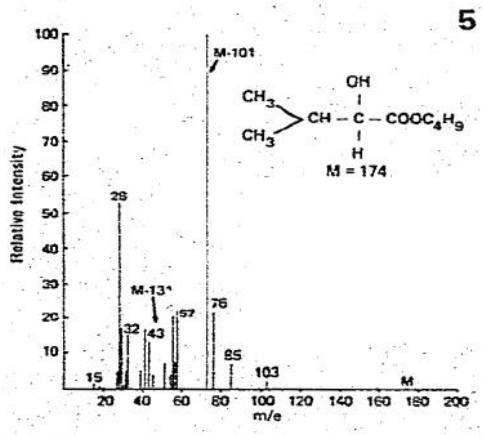
M-101
M-131
CH_3
CH_3
C(OH) $COOC_4H_9$
M = 160
Relative Intensity
m/e


5
M-101
M-131
CH_3
CH_3
CH − C(OH)H − $COOC_4H_9$
M = 174
Relative Intensity
m/e

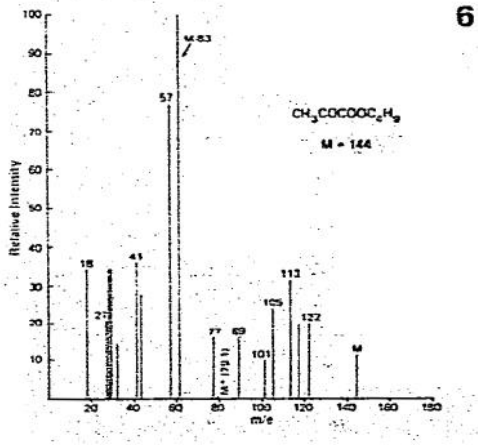

6
M-83
$CH_3COCOOC_4H_9$
M = 144
Relative Intensity
m/e

Fig. 1.

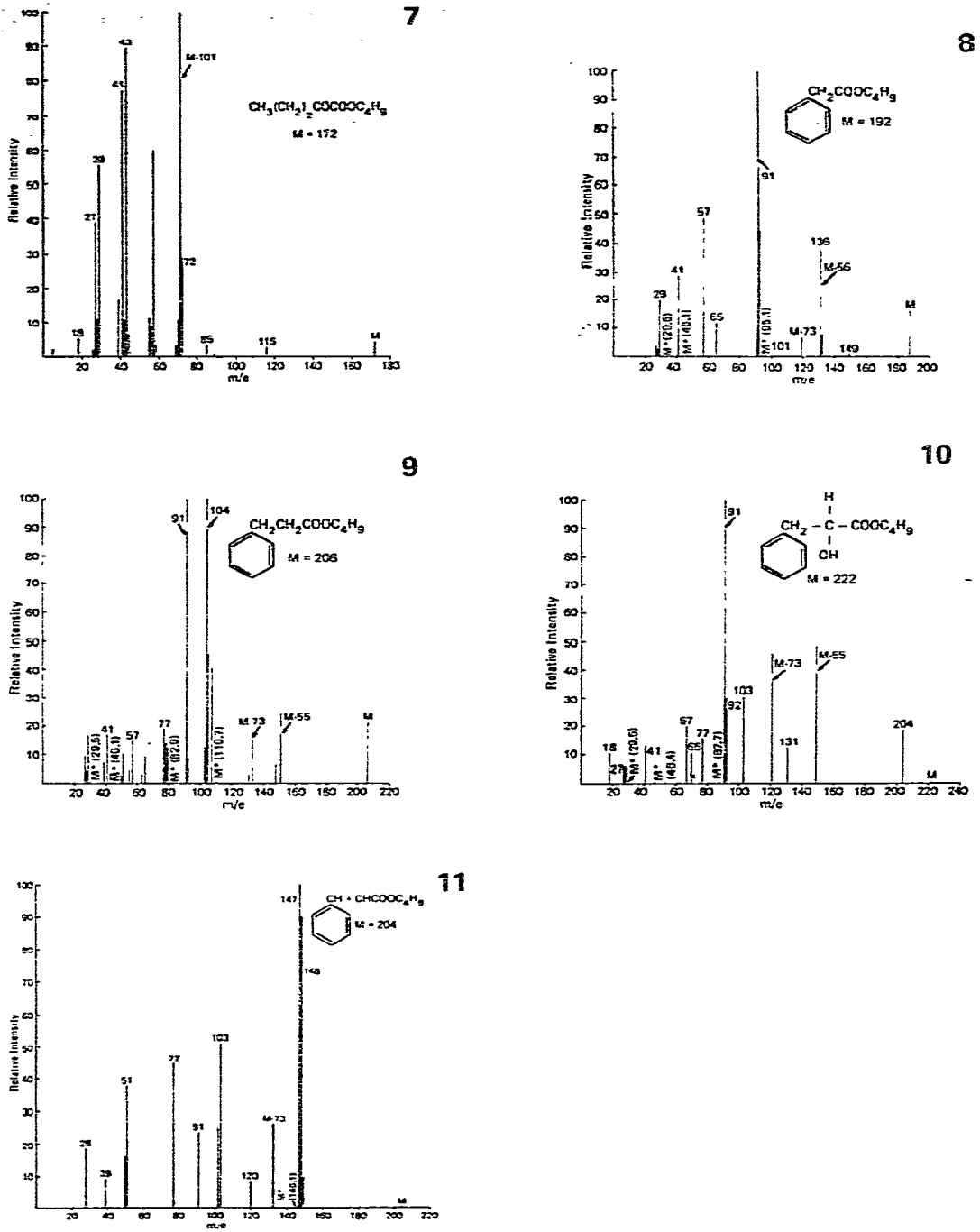


Fig. 1. Mass spectra of the butyl esters of the short chain acids. (1) Pentanoic acid; (2) 3-methylbutanoic acid; (3) 2-hydroxypropanoic acid; (4) 2-hydroxy-2-methylpropanoic acid; (5) 2-hydroxy-3-methylbutanoic acid; (6) 2-ketopropanoic acid; (7) 2-ketopentanoic acid; (8) phenylethanoic acid; (9) hydrocinnamic acid; (10) 2-hydroxy-3-phenylpropanoic acid; (11) cinnamic acid.

to butyl acetate[7,8], butyl benzoate and butyl propionate[9], and no systematic investigation of the fragmentation pattern of other *n*-BE of short chain acids has been reported. In this paper we have used the GLC–MS technique to determine the mass spectra of *n*-BE derivatives of short chain acids which we observe in our studies with microorganisms and body fluids[10,11]. The acids selected for study are straight and branched chain acids (C_2–C_8), α-hydroxy short chain acids, aliphatic α-keto acids, and some simple aromatic acids. We also identified two unknown acids present in a bacteriological culture medium using this technique.

EXPERIMENTAL*

The acids were obtained from Chem Service (Media, Pa., U.S.A.), Eastman-Kodak (Rochester, N.Y., U.S.A.) and Aldrich (Milwaukee, Wisc., U.S.A.). The acids were converted to butyl esters with 14% w/v boron trifluoride–butanol reagent (Applied Science, State College, Pa., U.S.A.) according to the procedure described previously[5].

GLC–MS of the *n*-BE was carried out on an LKB 9000 instrument. The mass spectra were recorded at an electron energy of 70 eV, a trap current of 60 μA, and an acceleration voltage of 3.5 kV. The ion source temperature was 290°, and the molecular separator temperature was 250°. The resolution was 1,000. The butyl esters were separated on a 0.16-in. (4.06-mm) I.D. × 12-ft. (3.66 m) glass column packed with 3% OV-1 coated on 80–100 mesh Chromosorb W (Applied Science). The column was operated isothermally at 190° for 5 min after the sample was injected; then it was programmed to 270° at a rate of 4°/min. Helium was used as carrier gas at a flow-rate of 35 ml/min.

RESULTS AND DISCUSSION

Representative mass spectra of the *n*-BE derivatives of straight and methyl branched saturated acids, straight and methyl branched 2-hydroxy acids, 2-keto acids and some simple aromatic short chain acids are shown in Fig. 1. The molecular ions were discernible only in the spectra of the keto and the aromatic acid esters. The presence of the dominating fragment ions at *m/e* M − 73, M − 55 and M − 101 and the base peak at *m/e* M − 73 in the spectra of saturated acids conforms with similar findings[7–9]. The mass spectra of 2-hydroxy and 2-keto acid esters showed the base peak at *m/e* M − 101 with one exception, that being, the base peak at *m/e* M − 83. Both hydroxy and keto esters also showed the presence of distinct fragment ions at *m/e* 57 and at M − 131 in their spectra. The main spectra of the esters of the keto acids showed less major ion fragmentations than the 2-hydroxy acids. As expected, all the aromatic acid esters investigated displayed a prominent fragment ion at *m/e* 91 (tropylium ion) which constituted the base peak in most instances.

The MS investigation of the *n*-BE of short chain acids and their major ion fragmentations are summarized in the following steps.

* Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

Saturated straight and branched chain acids

(a) Seven straight chain (C_2–C_8) and four methyl branched (C_2–C_4) acids were investigated.

(b) Cleavage of butoxy radicals from the molecular ions appears to produce the base peaks at *m/e* M − 73.

(c) Ions at *m/e* M − 101 are probably produced by the α-cleavage of the acid alkyl group (R) from the molecular ions. Ions at *m/e* M − 55 logically result from a similar cleavage of a butyl group followed by a double hydrogen rearrangement[12] giving $RCOOH_2^+$.

(d) The butyl ion $C_4H_9^+$ cleaved from the molecular ions possibly leads to the ions at *m/e* 56 ($C_4H_8^+$) and at *m/e* 41 ($C_3H_5^+$) resulting from a site specific McLafferty type rearrangement[13] and a loss of methyl group, respectively.

2-Hydroxy acid butyl esters

(a) Tree hydroxy acids, namely, 2-hydroxypropanoic acid, 2-hydroxy-2-methylpropanoic acid and 2-hydroxy-3-methylbutanoic acid were investigated.

(b) Base peak at *m/e* M − 101 in each case is believed to result from the similar α-cleavage of the molecular ion as described above in saturated acid esters.

(c) The other characteristic ions at *m/e* M − 131 are probably produced by a simple cleavage of the C–C bond adjacent to the carbon containing the hydroxyl group.

(d) The prominent ion at *m/e* 57 in each spectrum appears to be due to $C_3H_5O^+$ and this is confirmed by the observed metastable transition.

2-Keto acid butyl esters

(a) Two keto acids, namely, 2-ketopropanoic and 2-ketopentanoic acids were investigated.

(b) The base peaks at *m/e* M − 83 in one spectrum and at *m/e* M − 101 in the other suggest that the position of the keto group governs the MS fragmentation process. These peaks appear to result either from a simple cleavage of the molecular ion or from a rearrangement of the simple cleavage fragments. Thus the peak at *m/e* 61 (M − 83) in the spectrum of 2-ketopropanoic acid ester is possibly due to the cleavage of the OC_4H_9 radical from the molecular ion followed by double hydrogen rearrangement producing CH_3–C(OH)=$^+$OH. The M − 101 peak in the spectrum of 2-ketopentanoic acid ester is logically produced by the same simple cleavage as described above.

Simple aromatic acid butyl esters

(a) Five aromatic acids, namely phenylethanoic acid, hydrocinnamic acid, 2-hydroxy-3-phenylpropanoic acid, cinnamic acid and benzoic acid were studied. Our results for the *n*-BE of benzoic acid were in good agreement with that of McLafferty and Golke[14].

(b) A major ion at *m/e* 104 in the mass spectrum of hydrocinnamic acid ester appears to be due to Ph–$\dot{C}$H–$^+$$CH_2$ which is produced by the cleavage of $COOC_4H_9$ from the molecular ion followed by a single hydrogen rearrangement. A similar intense peak at *m/e* 104 has also been observed in the mass spectrum of the methyl ester of this acid[15].

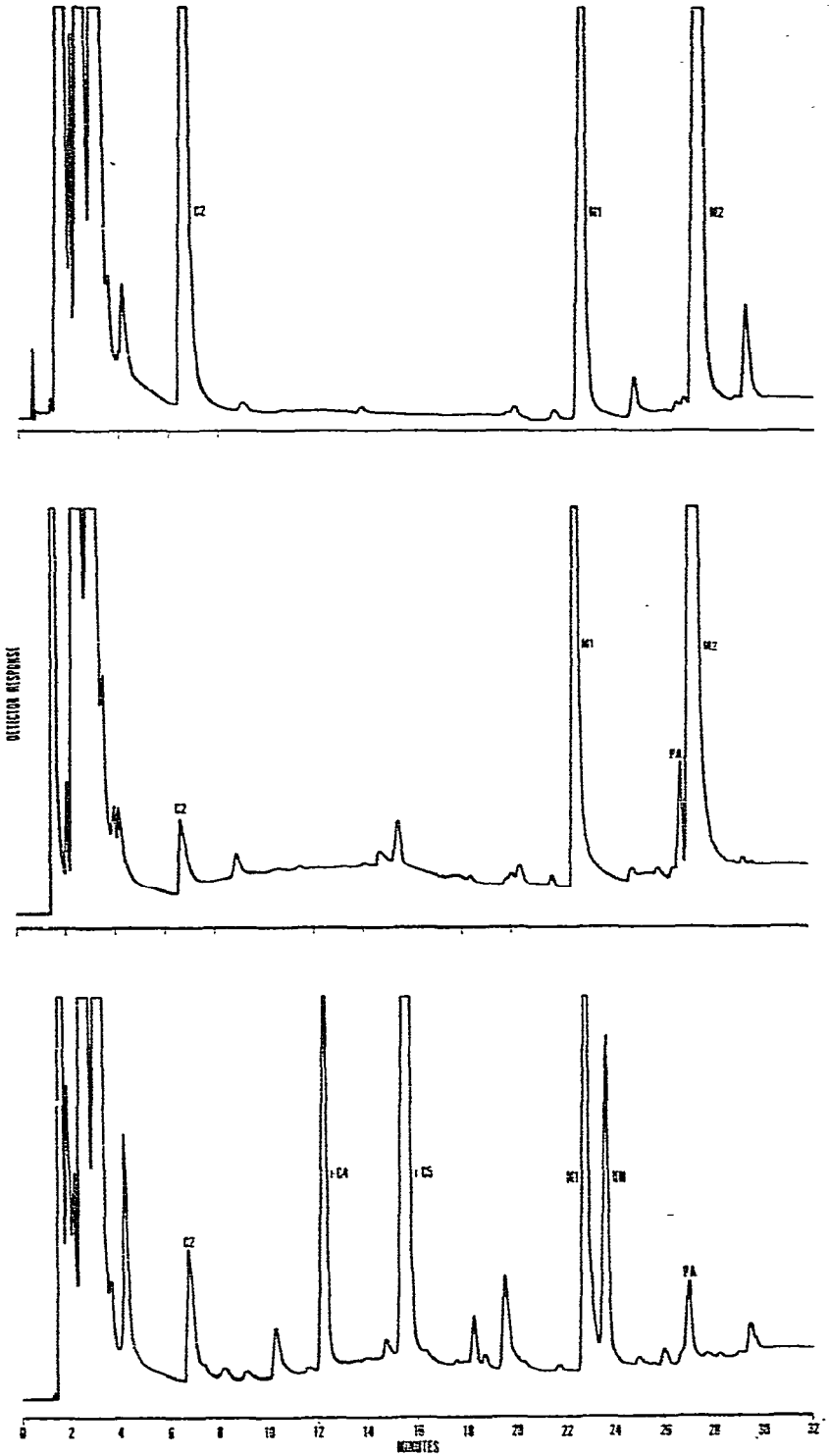


Fig. 2. Gas chromatograms of esterified short chain acids from *P. pseudoalcaligenes* (bottom), *P. alcaligenes* (center) and uninoculated Trypticase Soy Agar (top). C2, i-C4, i-C5 and PA refer to ethanoic, 2-methylbutanoic, 2-methylpentanoic and phenylethanoic acids, respectively. UN is an unidentified compound; M1 and M2 are media components.

(c) Cleavage of the butyl radical from the cinnamic acid ester parent ion probably produces the major fragment at *m/e* 147.

(d) All the aromatic acids investigated showed prominent fragment ions at *m/e* M — 73 and at *m/e* M — 101.

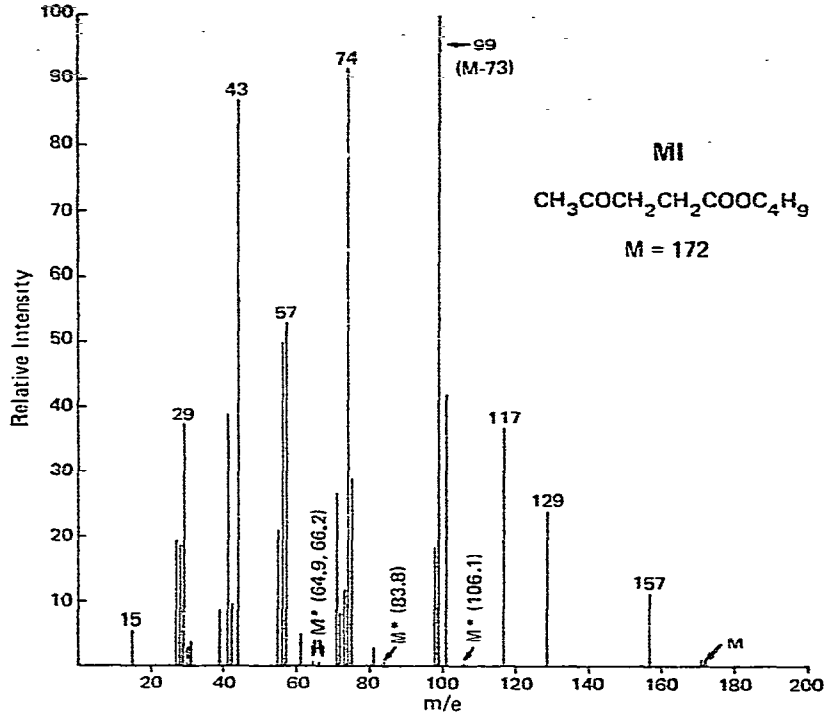
The results suggest that the mass spectra of *n*-BE of various classes of short chain acids can be identified by the presence of the simple cleavage fragment ions such as $M^+ - 73$ and $M^+ - 101$ or by the presence of rearrangement ions $M^+ - 55$ or $M^+ - 56$ in their spectra. The prominence of these ions in the spectrum depends upon the chemical structure of the compound. A branched methyl group within the molecule does not appear to affect the MS cracking pattern to any significant extent. Similar results regarding the effect of a branched methyl chain on the mass spectra were observed by Ryhage and Stenhagen[16] during their investigation of methyl esters of carboxylic acids. The fragment ions due to the classical McLafferty rearrangement[12] which invariably accounts for the base peaks in the spectra of methyl esters of carboxylic acids appear to be of little importance in the case of *n*-BE.

An example of our use of combined GLC–MS for analysis of short chain acids from microorganisms after growth in common laboratory media is illustrated in Fig. 2. From comparison of the chromatograms, it is quite apparent that there are clear, distinct differences between the two species. The bottom chromatogram shows that 2-methyl butanoic and 2-methyl pentanoic acids are the major compounds produced by *Pseudomonas pseudoalcaligenes*, whereas these two acids were absent or present in only trace amounts in *P. alcaligenes* (center). An additional difference between the two species was the presence of a peak at 23.5 min (unidentified compound) in *P. pseudoalcaligenes* which was consistently absent in *P. alcaligenes*. Identification of this component has not been completed. Both species produced relatively small amounts of phenyl ethanoic acid (PA). The top chromatogram was obtained with a sample of the uninoculated control Trypticase Soy Agar (TSA) medium and shows three major peaks labeled C2, M1, and M2. The identity of the C2 peak was readily established by combined GLC–MS as ethanoic acid. It is clear from comparison of the chromatograms that both bacterial species were able to metabolize one or more of the components in the control media as evidenced by a substantial decrease in peak areas. The size of the ethanoic acid peak was markedly reduced in both cultures, whereas the M2 peak was completely absent in the *P. pseudoalcaligenes* cultures. These observations prompted further study to identify M1 and M2.

On the basis of solubility characteristics and ease of conversion to esters under normal esterification procedures, M1 and M2 appeared to be acids. However, several common acids (as *n*-BE derivatives) failed to give matching GLC retention times. The mass spectra of the *n*-BE derivatives of M1 and M2 were obtained and are presented in Fig. 3. Both compounds showed a molecular ion and, like all other acid esters, a large M — 73 ion resulting from simple cleavage of the molecular ion, as discussed previously. Careful study of the fragmentation pattern indicated that M1 was the *n*-BE derivative of 4-ketopentanoic acid. Metastable ions which were present at *m/e* 64.9, 66.2, 83.9, and 106.1 account for the majority of the major ions.

A relatively intense ion at *m/e* 74 was also present in the spectrum and is thought to represent the ion fragment $[C_4H_9OH]^+$. This ion could result from cleavage of OC_4H_9 from the molecular ion followed by a single hydrogen rearrangement.

The *n*-BE derivatives of authentic standards of 2, 3, and 4-ketopentanoic



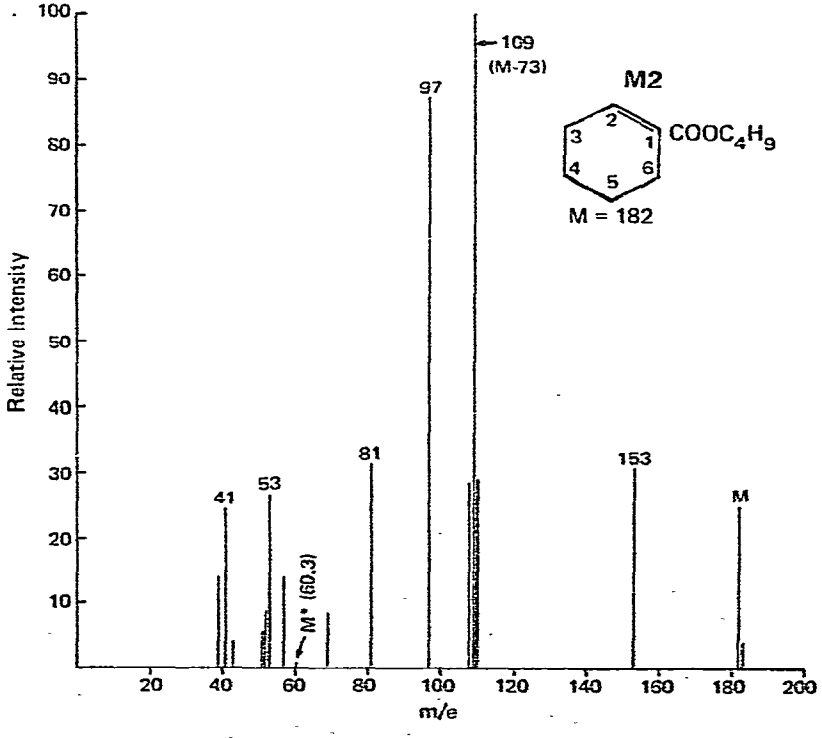


Fig. 3. Mass spectra of the butyl esters of media components (M1 and M2) from Trypticase Soy Agar. Top, 4-ketopentanoic acid; bottom, cyclohexene-1-carboxylic acid.

acids were prepared, and their GLC characteristics and mass spectra were compared to M1. The 4-keto acid was identical in all respects to M1, whereas the 2 and 3-keto isomers gave different GLC retention times and different mass spectra.

Thorough examination of the mass spectrum of M2 suggested a possible structure to be cyclohexene-1-carboxylic acid. All the expected major ions for this compound (*m/e* 41, 53, 81, 97, 109) are present in the spectrum (Fig. 3). These ions can be accounted for by a retro Diels–Alder type cleavage accompanied by a single hydrogen rearrangement in the initial step. The fragmentation appears to occur according to Fig. 4.

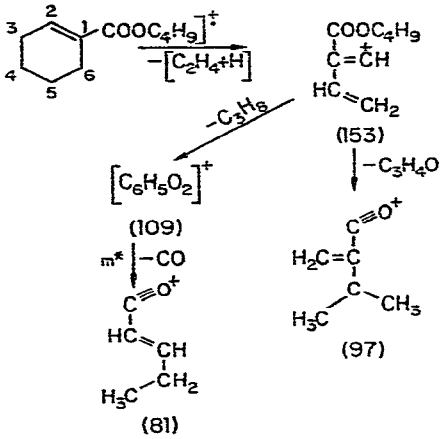


Fig. 4. Fragmentation pattern of M2 (see Figs. 2 and 3).

The presence of a metastable peak at *m/e* 60.3 would account for the *m/e* 109 to *m/e* 81 transition. A standard of cyclohexene-1-carboxylic acid was not available for comparison by GLC and MS; however, upon hydrogenation[17] the M2 peak disappeared from the chromatogram, and a new peak appeared with a GLC retention time identical to that of an authentic standard of cyclohexane carboxylic acid (as the *n*-BE derivative). Moreover, the mass spectrum of the hydrogenated M2 peak was identical to that of cyclohexane carboxylic acid.

To our knowledge 4-ketopentanoic acid (M1) and cyclohexene-1-carboxylic acid (M2) are not common components of most bacteriological media. No data are available at this time to account for their presence in the TSA medium. The source of these acids and the processes involved in their metabolism by various *Pseudomonas* species require additional study.

ACKNOWLEDGEMENTS

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