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GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF DI-, KETO- AND HYDROXYCARBOXYLIC ACID BENZYL ESTERS

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SUMMARY

The benzyl esters of di-(C_2–C_6)- and hydroxycarboxylic acids were prepared by reaction with N,N'-dicyclohexyl-O-benzylisourea or with phenyldiazomethane. The hydroxycarboxylic acids lactic and 3-hydroxybutyric acid were also alkylated with N,N'-dicyclohexyl-O-benzylisourea to give the corresponding O-benzyl ethers. The 2-oxocarboxylic acids pyruvic, oxaloacetic and 2-oxoglutaric acid were converted by means of O-benzylhydroxylamine hydrochloride into the corresponding O-benzyl oximes prior to esterification with phenyldiazomethane. The carboxylic acid benzyl esters were separated by gas chromatography on an SE-30 column. Mass spectra of the compounds were recorded with a MAT 111 GC–MS system. The main fragments at *m/e* 91 and *m/e* 107, found in all mass spectra, were due to the benzyl alcohol moiety of the esters. The general fragmentation pattern was similar to that of fatty acid and aromatic acid benzyl esters. The fragmentation scheme for this class of substances is discussed.

INTRODUCTION

Gas chromatographic–mass spectrometric (GC–MS) analysis of fatty acid benzyl esters has been described by us previously[1,2]. The method was proved to be particularly suitable for the gas–liquid chromatographical (GLC) separation of short and medium chain fatty acids[1]. Thus, for short chain fatty acids, an analytical procedure was developed that was as useful as the methylation method applied in the GLC of long chain fatty acids.

Despite continuous investigations[3–5] on the GLC separation of di- and polyfunctional carboxylic acids, difficulties remained and no standard method has been developed hitherto. The two derivatives of carboxylic acids most frequently used for GLC were the methyl and trimethylsilyl esters. Difficulties arose essentially from the preparation of the esters, because of the formation of by-products. Diazomethylation, for example, resulted in numerous compounds that interfered in gas chromatograms[4]. In particular, the 2-oxocarboxylic acids reacted to several products owing to the enolization of the 2-oxo group.

In this work, benzylation by means of phenyldiazomethane and N,N'-dicyclo-

hexyl-O-benzylisourea was extended to di- and polyfunctional carboxylic acids. Analytical investigations of these acids are of particular interest with regard to their biological occurence in the citrate cycle and related metabolic reactions. Various mixtures of carboxylic acid benzyl esters were separated by gas chromatography and the efficiency of the method was demonstrated.

After the GLC separations, mass spectra were recorded. The methyl esters of these acids have been analyzed systematically by mass spectrometry[4], while the benzyl esters were still unexplored in this respect. As expected, the mass spectra of these compounds showed the characteristic fragmentation pattern of benzyl esters reported for fatty acid benzyl esters. The mass spectra, however, were more complicated because of the additional functional groups present in the carboxylic acids investigated.

MATERIALS

Lactic, DL-3-hydroxybutyric, malic, 2-oxoglutaric, oxaloacetic, malonic, succinic, glutaric, adipic and maleic acids were obtained from E. Merck (Darmstadt, G.F.R.) and pyruvic and fumaric acids from Fluka (Buchs, Switzerland). All solvents (reagent grade) and chemicals were purchased from E. Merck.

The gas–liquid chromatograph (Model 402, Hewlett-Packard, Palo Alto, Calif., U.S.A.) was equipped with a flame ionization detector and recorder (Hewlett-Packard, Model 7127 A). Glass columns (1.70 m × 3 mm I.D.) were packed with 3% SE-30 on Chromosorb Q, 100–120 mesh, obtained from Applied Science Labs. (State College, Pa., U.S.A.). For GC–MS measurements, a MAT 111 GC–MS system (Varian-MAT, Bremen, G.F.R.) was used. Mass spectra were recorded with an Oscilloport light-point recorder (Siemens, Erlangen, G.F.R.). GC separations were achieved on a glass column (9 m × 2 mm I.D.) packed with 3% OV-101 on Gas-Chrom Q, 100–120 mesh (Serva, Heidelberg, G.F.R.).

METHODS

Preparation of carboxylic acid benzyl esters

Carboxylic acid benzyl esters were synthesized by esterification of carboxylic acids with phenyldiazomethane (PDM) or N,N'-dicyclohexyl-O-benzylisourea (DBI)[1]. All esters were prepared in chloroform–methanol (2:1, v/v). Hydroxy- and 2-oxocarboxylic acids were treated with PDM at 0°.

Preparation of benzoximes of 2-oxocarboxylic acids

The benzoximes were prepared in a similar manner to the analytical procedure described by Chalmers and Watts[6]. A 3-mmole amount of 2-oxocarboxylic acid and a four-fold excess of O-benzylhydroxylamine hydrochloride were stirred overnight in 50 ml of anhydrous pyridine. The solvent was removed by evaporation under reduced pressure, and the residue was dissolved in 100 ml of 1 *N* hydrochloric acid. The clear solution was extracted five times with 60-ml portions of diethyl ether and the combined ether extracts were evaporated to dryness. The crude product was redissolved in 50 ml of diethyl ether and dried for 3 h over anhydrous sodium sulphate.

The solution was filtered, the solvent removed and the crystalline benzoximes were washed with small volumes of benzene and dried over anhydrous calcium chloride.

Gas chromatography of carboxylic acid benzyl esters

The carboxylic acid benzyl esters were separated on an SE-30 column. Identical flow-rates were used for all separations: helium at 60 ml/min, synthetic air at 350 ml/min and hydrogen at 38 ml/min. For temperature programming, the temperatures of the injection port and the flame ionization detector were adjusted so as to be 30° above the final temperature.

GC–MS of single carboxylic acid benzyl esters

Carboxylic acid benzyl esters (2 μl of a 1% *n*-pentane solution) were separated from the solvent with the MAT 111 GC–MS system and the mass spectra were recorded using the following conditions: ionizing voltage, 80 V; current, 270 μA; ion source temperature, 300°; inlet tube temperature, 220°. Depending on the volatility of the carboxylic acid benzyl esters, different column and injection port temperatures were used.

Evaluation of mass spectra

The intensities of fragments were measured relative to the peak with the greatest intensity (100%). All peaks in the range *m/e* 40–250 that were at least 1% of the intensity of the base peak were plotted.

RESULTS

Esterification with PDM

Saturated dicarboxylic acids, hydroxycarboxylic acids, maleic acid and the benzoximes of the 2-oxocarboxylic acids gave almost quantitatively the benzyl esters upon esterification with PDM. The reaction of hydroxycarboxylic acids without the formation of by-products, however, was successful only at 0°. Fumaric acid formed the corresponding 4,5-dicarbobenzoxy-3-phenylpyrazoline with PDM. It was impossible, by esterification with PDM, to convert 2-oxocarboxylic acids, such as pyruvic, oxaloacetic and 2-oxoglutaric acids, into their benzyl esters. With different concentrations of the reagents and temperatures, a number of unidentified compounds always appeared.

Esterification with DBI

Most of the carboxylic acids investigated could be esterified upon treatment with DBI as alkylating agent, especially by reactions in boiling benzene or dioxan. 2-Oxoglutaric acid, oxaloacetic acid and their 2-benzoximes gave no benzyl esters. Lactic acid and 3-hydroxybutyric acid were esterified and additionally alkylated at their hydroxy function, leading to benzoxy ethers.

Gas–liquid chromatography

Fig. 1 shows the temperature-programmed separation of the benzyl esters of seven dicarboxylic acids simultaneously esterified with PDM.

Benzylation with PDM gave, in addition to the reaction artefact A, no com-

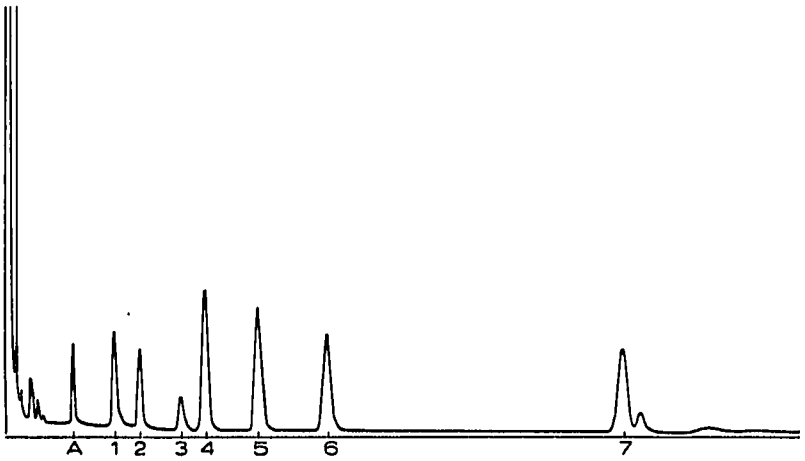

Fig. 1. Chromatogram of seven carboxylic acid benzyl esters on an SE-30 column programmed from 180° to 250° at 2°/min. A, Reaction artefact; 1, oxalic acid dibenzyl ester; 2, malonic acid dibenzyl ester; 3, maleic acid dibenzyl ester; 4, succinic acid dibenzyl ester; 5, glutaric acid dibenzyl ester; 6, adipic acid dibenzyl ester; 7, 4,5-dicarbobenzoxy-3-phenylpyrazoline.

pounds that showed interfering peaks in the range of the carboxylic acid benzyl esters. Low-boiling by-products appeared only between the solvent peak and the reaction artefact. No fumaric acid dibenzyl ester was formed, the pyrazoline peak being the last in the chromatogram. All peaks were well separated. Owing to the different volatilities of the compounds, it was not possible to run the gas chromatogram isotherm.

The separation of a mixture of benzyl esters of eleven carboxylic acids from

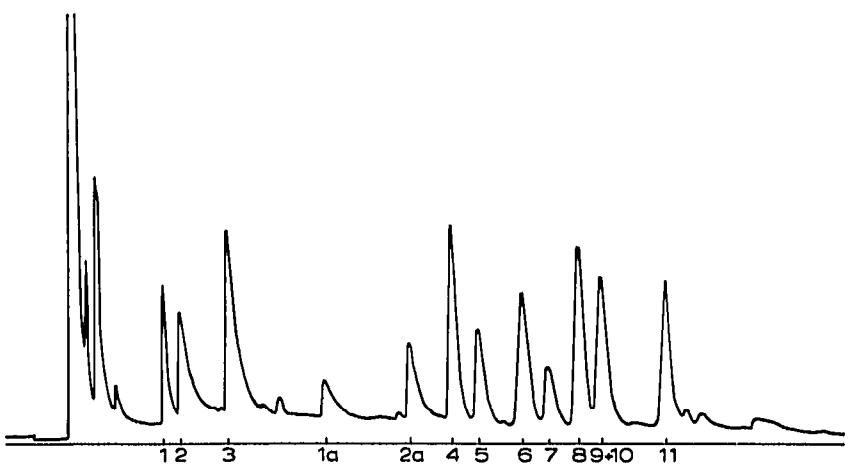

Fig. 2. Chromatogram of eleven carboxylic acid benzyl esters on an SE-30 column programmed from 95 to 185° at 5°/min, then from 185° to 220° at 2°/min. 1, Lactic acid benzyl ester; 2, pyruvic acid benzyl ester; 3, 3-hydroxybutyric acid benzyl ester; 4, oxalic acid dibenzyl ester; 5, malonic acid dibenzyl ester; 6, maleic acid dibenzyl ester; 7, succinic acid dibenzyl ester; 8, fumaric acid dibenzyl ester; 9, glutaric acid dibenzyl ester; 10, malic acid dibenzyl ester; 11, adipic acid dibenzyl ester; 1a, lactic acid benzyl ester O-benzyl ether; 2a, 3-hydroxybutyric acid benzyl ester O-benzyl ether.

lactic to adipic acid is shown in Fig. 2. All esters were prepared by the DBI method. Malic acid showed the same retention time as glutaric acid dibenzyl ester. The separation of these two esters could not be achieved, either by another temperature programme or by using EGSS-X as the stationary phase. The intensity of some interfering peaks in the chromatogram was very small compared with the intensity of the benzyl esters. Impurities of higher concentrations appeared only in the range between the solvent peak and the lactic acid benzyl ester peak. The benzyl esters and the benzyl ester O-benzyl ethers of the hydroxycarboxylic acids gave considerable tailing on the SE-30 column, the peaks of the other esters being satisfactorily separated. The three 2-oxocarboxylic acids (pyruvic, oxaloacetic and 2-oxoglutaric acids) were converted into the corresponding O-benzyloximes by means of O-benzylhydroxylamine, and separated by gas chromatography as their benzyl esters from a mixture of carboxylic acid benzyl esters (Fig. 3). Pyruvic acid benzoxime benzyl ester could not be separated from malonic acid benzyl ester.

Mass spectrometry

Fig. 4 shows the mass spectra of the benzyl esters of lactic, 3-hydroxybutyric, malic, oxalic, succinic, glutaric and adipic acids. Spectra could not be obtained for the benzyl esters of malonic, fumaric, maleic and the 2-oxocarboxylic acids. The mass spectrum of malic acid benzyl ester showed only a few characteristic peaks and could not be interpreted.

Lactic acid and 3-hydroxybutyric acid gave a molecular ion with 5% relative intensity. The other esters could be detected by their $M^+ - 91$ fragments, which

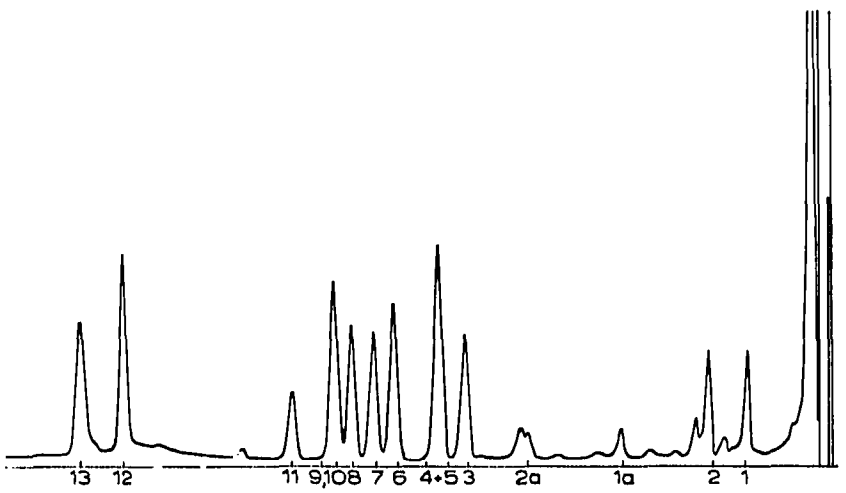

Fig. 3. Chromatogram of thirteen carboxylic acid benzyl esters on an SE-30 column programmed from 144° to 257° at 3°/min, then from 257° to 290° at 5°/min and isothermal at 290°. 1, Lactic acid benzyl ester; 2, 3-hydroxybutyric acid benzyl ester; 3, oxalic acid dibenzyl ester; 4, malonic acid dibenzyl ester; 5, pyruvic acid benzyl ester benzoxime; 6, maleic acid dibenzyl ester; 7, succinic acid dibenzyl ester; 8, fumaric acid dibenzyl ester; 9, glutaric acid dibenzyl ester; 10, malic acid dibenzyl ester; 11, adipic acid dibenzyl ester; 12, 2-oxoglutaric acid dibenzyl ester benzoxime; 13, oxaloacetic acid dibenzyl ester benzoxime; 1a, lactic acid benzyl ester O-benzyl ether; 2a, 3-hydroxybutyric acid benzyl ester O-benzyl ether.

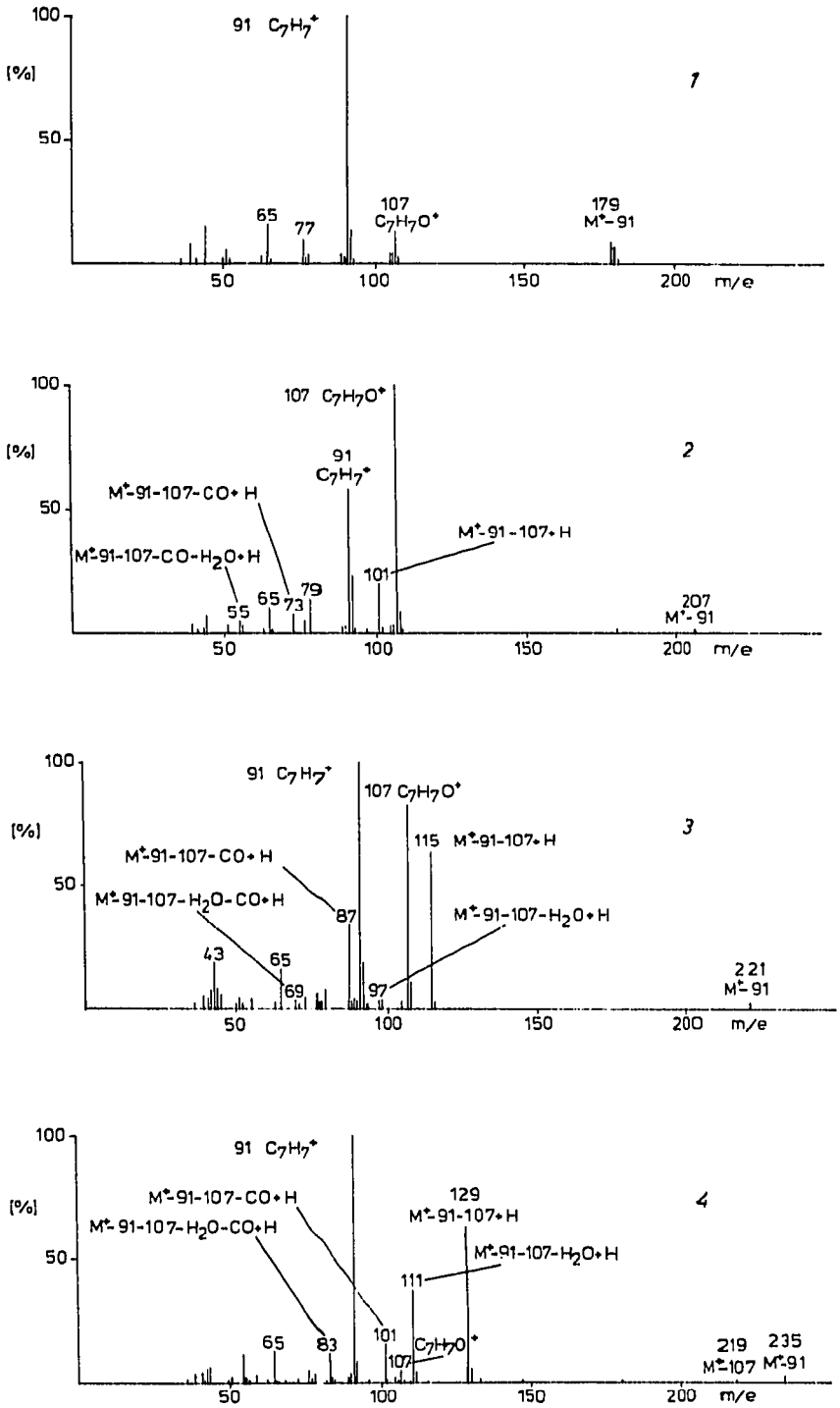
100
[%]
50
91 $C_7H_7^+$
65
77
107
$C_7H_7O^+$
179
M^+-91
1
50
100
150
200 m/e
107 $C_7H_7O^+$
91
$C_7H_7^+$
M^+-91-107-CO+H
M^+-91-107+H
M^+-91-107-CO-H_2O+H
55
65
73
79
101
207
M^+-91
2
91 $C_7H_7^+$
107 $C_7H_7O^+$
115 M^+-91-107+H
M^+-91-107-CO+H
M^+-91-107-H_2O-CO+H
M^+-91-107-H_2O+H
87
43
65
69
97
221
M^+-91
3
91 $C_7H_7^+$
M^+-91-107-CO+H
M^+-91-107-H_2O-CO+H
129
M^+-91-107+H
M^+-91-107-H_2O+H
111
65
83
101
107
$C_7H_7O^+$
219
M^+-107
235
M^+-91
4

Fig. 4.

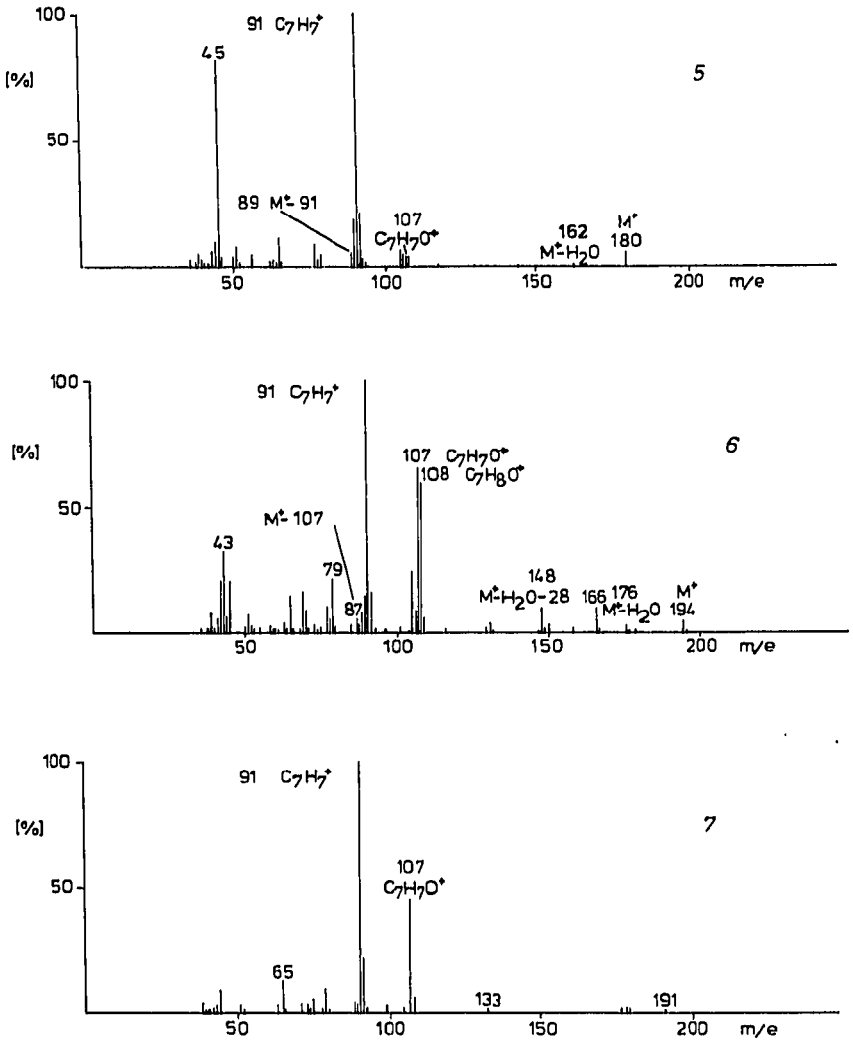

Fig. 4. Mass spectra of carboxylic acid benzyl esters. 1, Oxalic acid dibenzyl ester; 2, succinic acid dibenzyl ester; 3, glutaric acid dibenzyl ester; 4, adipic acid dibenzyl ester; 5, lactic acid benzyl ester; 6, 3-hydroxybutyric acid benzyl ester; 7, malic acid dibenzyl ester.

varied in intensity from 2 to 15%. Species of *m/e* 91 (tropylium) and *m/e* 107 (benzoxy radical cation) appeared in all mass spectra with high intensities. The species of *m/e* 107 was the base peak in the succinic acid benzyl ester spectrum, *m/e* 91 in all others. Tropylium and phenyl cations decomposed and gave the known aromatic fragments. $M^+ - 107$, produced by cleavage of the benzoxy radical from the molecular ion, appeared only in the spectrum of 3-hydroxybutyric acid and adipic acid benzyl ester. With hydroxycarboxylic acid benzyl esters, the cleavage of H_2O from M^+ to give $M^+ - 18$ was characteristic. For the decomposition of carboxylic acid benzyl esters, the following steps are suggested.

(1) Dicarboxylic acid benzyl esters:

(a) Cleavage of benzyl radicals from M^+ led to carboxyl cations ($M^+ - 91$).

(b) Cleavage of benzoxy radicals from the molecular ion produced acyl cations ($M^+ - 107$).

(c) Cleavage of benzyl radicals and benzoxy radicals with simultaneous capture of a hydrogen atom gave acyl carboxyl cations ($M^+ - 91 - 107 + H$).

(d) Cleavage of H_2O from acyl carboxyl cations produced acyl aldoketene cations ($M^+ - 91 - 107 - H_2O + H$).

(e) Cleavage of CO from acyl carboxyl cations produced alkylcarboxylic acid cations ($M^+ - 91 - 107 - CO + H$).

(f) Cleavage of CO from acyl aldoketene cations gave aldoketene cations ($M^+ - 91 - 107 - H_2O - CO + H$).

(g) Cleavage of H_2O from alkyl carboxylic acid cations led to the same aldoketene cations ($M^+ - 91 - 107 - CO - H_2O + H$).

(h) By cleavage of acyl radicals from M^+, the benzoxy radical cation at *m/e* 107 was formed.

(i) Cleavage of carboxylic radicals from M^+ produced a tropylium cation of *m/e* 91, which led to the fragments of *m/e* 65 and *m/e* 39.

(2) Hydroxycarboxylic acid benzyl esters:

The decomposition steps were the same as proposed in steps (a), (b), (h) and (i) for the dicarboxylic acid benzyl esters. However, some other fragments also appeared in these spectra, as follows.

(j) Cleavage of H_2O from the molecular ion produced a vinyl radical cation ($M^+ - H_2O$).

(k) Cleavage of hydroxyaldoketenes from M^+ and rearrangement of an H atom gave the benzyl alcohol radical cation of *m/e* 108.

(l) A fragment $M^+ - H_2O - 28$ was observed in the spectrum of 3-hydroxybutyric acid benzyl ester.

In no case could a cleavage of two tropylium cations or two benzoxy cations be observed.

DISCUSSION

The preparation of carboxylic acid benzyl esters by reaction of carboxylic acids with PDM or DBI as alkylating agents is a simple method which can be applied to many acids, and which converts the non-volatile carboxylic acids into derivatives suitable for gas chromatographic studies.

Esterification of saturated dicarboxylic acids, hydroxycarboxylic acids and maleic acid was achieved with both reagents without problems. Fumaric acid gave a pyrazoline derivative with PDM. An analogous reaction has been described by several workers in connection with the treatment of fumaric acid with diazomethane[3,7]. 2-Oxocarboxylic acids did not give benzyl esters in satisfactory yields with either PDM or with DBI. Pure pyruvic acid benzyl ester could be obtained in a yield of only 20% after successive column chromatography on silica gel. The high reactivity of the 2-oxo function was responsible for the formation of by-products, which differed between acids in both number and amount. Among others, glycidates and 2,2-

dibenzoxy derivatives were formed[4]. 2-Hydroxy and 3-hydroxy acids were alkylated by DBI not only at the carboxylic group but also at the hydroxy function. Vowinkel[8], who introduced this method, reported only alkylation of phenolic hydroxy functions, O-alkylation at unprotected alcoholic hydroxy functions not being observed.

The results for the 2-oxocarboxylic acids could be improved by converting the 2-oxo function with O-benzylhydroxylamine into a 2-benzoxime. Benzylation of the protected 2-oxoacid with PDM was then successful. The retention times of the 2-oxocarboxylic acid benzoxime benzyl esters did not interfere with the peaks of the other carboxylic acid benzyl esters in the gas chromatogram. Only pyruvic acid benzoxime benzyl ester and malonic acid dibenzyl ester exhibited the same retention time. The benzoximes of the 2-oxodicarboxylic acid dibenzyl esters were less volatile than the dibenzyl esters of the dicarboxylic acids and well separated from them.

The mass spectra, especially the dominating peaks of *m/e* 91, *m/e* 107 (108) and the $M^+ - 91$ fragment, were similiar to the spectra of benzyl esters described by Emery[9] and the spectra of fatty acid benzyl esters investigated by Hintze *et al.*[2]. In addition to the cleavage of a benzyl fragment or a benzoxy (benzyl alcohol) fragment, which was also found in the spectra of fatty acid benzyl esters, dicarboxylic acid benzyl esters split off a second fragment of *m/e* 91 or *m/e* 107 (108). If the first group to be split off were a benzyl group, then the second was a benzoxy group, and *vice versa*. Cleavage of two *m/e* 91 or two *m/e* 107 (108) fragments was not observed for the esters investigated. Following the cleavage of two aromatic fragments, the successive splitting of H_2O and CO or of CO and H_2O led to the same molecule, whereby the intermediates increased in intensity as the number of carbon atoms in the ester increased. A break within the hydrocarbon chain, which was observed in long chain fatty acid benzyl esters and methyl esters, was found only for adipic acid benzyl ester. Products of McLafferty rearrangements did not appear, not even with carboxylic acid benzyl esters that have a γ-hydrogen atom. Peaks of protonated acids were missing from the spectra. In the hydroxycarboxylic acid benzyl esters, the cleavage of H_2O from M^+ was remarkably strong. The $M^+ - H_2O$ peaks were more intense than in the corresponding methyl esters investigated by Ryhage and Stenhagen[10]. The resulting α,β-unsaturated benzyl esters were well stabilized by mesomeric structures. The hydroxycarboxylic acid benzyl ester O-benzyl ethers were also identified by mass spectrometry. In addition to the fragments of *m/e* 91 and *m/e* 107 (108), however, no similarity with the spectra of the other benzyl esters in the fragmentation pattern was found.

Owing to the lability of the compounds, no spectra could be obtained from malonic acid benzyl ester, unsaturated carboxylic acid benzyl esters and 2-oxocarboxylic acid benzyl esters. Malonic acid was decarboxylated easily under the influence of heat and metal catalysts. The other acid derivatives underwent side-reactions with their additional functional groups, a problem that was also observed in the alkylation with PDM and DBI. The conditions found in the GC–MS separator, the presence of metal catalysts and high temperatures made the decomposition of these benzyl esters unavoidable.

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