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Short Communication

Identification of side-products formed by derivatization of 2-hydroxycarboxylic acids with methyl and ethyl chloroformate

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ABSTRACT

Derivatization of 2-hydroxycarboxylic acids with methyl and ethyl chloroformate leads to O-alkoxycarbonylalkyl esters. In addition to the main product a number of side-products are formed, which were identified by gas chromatography–mass spectrometry. They include lactides and "inter-ester" oligomers with alkoxycarbonyl-derivatized and with underivatized terminal OH group. The "inter-esters" result from the reaction between two, three or four hydroxycarboxylic acid molecules.

INTRODUCTION

2-Hydroxycarboxylic acids (HAs) are regular metabolites in blood serum and urine, and originate from glycolysis and degradation of the branched-chain amino acids valine, leucine and isoleucine. They can be determined in form of their methyl ester [1] or trimethylsilyl ester [2] derivatives. Alternatively, the 2-HAs are converted into O-alkoxycarbonylalkyl esters (R'OCOO–CHR–COOR') using methyl or ethyl chloroformate (MCF, ECF) [3]. However, the main reaction product is always accompanied by a number of side-products, the relative amounts of which are dependent on the reaction conditions chosen. In this work, the structures of the side-products obtained by derivatizing reference 2-HAs with MCF and ECF were examined by gas chromatography–mass spectrometry (GC–MS).

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EXPERIMENTAL

Chemicals

All solvents and reference substances were of the highest grade available. Methanol, ethanol, acetonitrile, pyridine and 0.05 *M* boric acid–0.05 *M* potassium chloride–0.02 *M* sodium hydroxide buffer (pH 9) were obtained from Merck (Darmstadt, Germany) and MCF, ECF and chloroform from Aldrich (Steinheim, Germany). The following reference substances were used: D-lactic acid, D,L-3-hydroxybutyric acid (Aldrich), 2-hydroxyisobutyric

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acid (Serva, Heidelberg, Germany), sodium D,L-2-hydroxybutyrate, D,L-2-hydroxyisovaleric acid (Fluka, Neu-Ulm, Germany), D,L-2-hydroxyvaleric acid, D,L-2-hydroxycaproic acid and L-2-hydroxyisocaproic acid (Sigma, Deisenhofen, Germany).

Derivatization procedure

Standard solutions were prepared for each 2-HA investigated at a concentration of 200 nmol/μl. In the reaction with MCF, 5 μl of each standard solution was mixed with 50 μl of diluted (1:6) buffer solution (pH 9), 5 μl of methanol, 145 μl of acetonitrile, 10 μl of MCF and 15 μl of pyridine. The reaction was completed after 1 min. After adding 150 μl of chloroform and 200 μl of a saturated aqueous sodium hydrogencarbonate solution, the reaction mixture was thoroughly shaken. The chloroform phase was separated, dried with anhydrous sodium sulphate and subjected to GC and GC–MS analysis. In the reaction with ECF, ethanol was substituted for methanol and ECF for MCF. In an additional experiment the standard solutions were reacted with ECF in the presence of methanol instead of ethanol.

Gas chromatographic and gas chromatographic–mass spectrometric analysis

The GC separations were performed on a Vega 6130 gas chromatograph with flame ionization detection (Carlo Erba, Hofheim, Germany) under the following conditions: 25 m × 0.25 mm I.D. fused-silica column coated with 0.25 μm OV-1701 (Macherey–Nagel, Düren, Germany); carrier gas, helium with a head pressure of 100 kPa; column temperature, programmed from 60 to 250°C at 10°C/min; injector block temperature, 240°C; detector temperature, 290°C; and sample size, 2 μl at a splitting ratio of 1:20.

The GC–MS analyses were carried out on a TSQ 70 system (Finnigan MAT, Munich, Germany). The column, carrier gas and sample size were the same as used for the GC separations. The column temperature was held at 60°C for 3 min, then programmed to 200°C at 5°C/min, to 230°C at 10°C/min and held at that temperature for 15 min. The temperatures were 250°C for the injector block, 250°C for the GC–MS transfer line and 150°C for the ion source. The mass spectrometer was run in the chemical ionization mode (CI) using methane as the reactant gas.

RESULTS AND DISCUSSION

When a mixture of reference 2-HAs is reacted with MCF according to the described conditions, a multitude of side-products are formed in addition the O-methoxycarbonylmethyl esters. The same holds true when using ECF as the derivatization agent. Mass spectrometric analysis reveals several series of reaction products for each 2-HA. The structures of these products are derived from the mass spectrometric data accumulated from the study of the homologous series of the individual 2-HAs, of pairs of 2-HAs and of the analogous derivatives obtained by varying the MCF, ECF, methanol and ethanol reagents. Reference substances for the side-products were not available.

The main product is the derivative intended to be prepared by the procedure, the O-methoxycarbonylhydroxycarboxylic acid methyl ester (MOC-HA-ME) or O-ethoxycarbonylhydroxycarboxylic acid ethyl ester (EOC-HA-ET), as shown in structure I in Fig. 1. One side-product is an "inter-ester" derivative, resulting from two hydroxycarboxylic acids, MOC-HA-HA-ME and the corresponding ethyl derivative (structure II in Fig. 1). Another side-product is an "inter-ester" including three 2-HA moieties, MOC-HA-HA-HA-ME (structure III in Fig. 1). Apparently, the formation of "inter-ester" oligomers is a general feature of the reaction between 2-HAs and MCF, with yields decreasing with the number of monomers involved. A further series of side-products of each 2-HA are probably the esters and "inter-esters" with the OH group underivatized (structure IV in Fig. 1). Finally, lactides are formed as side-products (structure V in Fig. 1).

The multitude of side-products is high because different 2-HAs present in mixtures of reference substances or in biological material inter-react with each other leading to a variety of mixed "inter-esters". The number of side-products when working with racemic reference substances is further increased through the formation of diastereomeric "inter-esters" and lactides.

As shown for D-lactic acid (Fig. 2), the major side-products in the reaction of 2-HAs with MCF are the "inter-esters" with two and three 2-HA moieties. The CI mass spectra of MOC-HA-ME and the side-products are characterized by typical general patterns. $[M+1]^+$ is an abundant ion in all

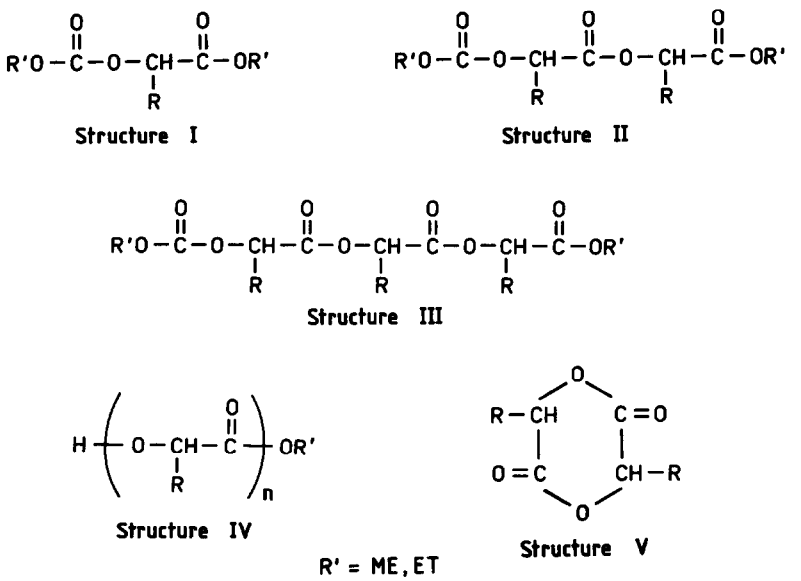

Fig. 1. Structures of the reaction products of 2-hydroxycarboxylic acids with methyl and ethyl chloroformate.

instances. In addition, the adduct ions $[M+29]^+$ and $[M+41]^+$ contribute to easy recognition of the molecular masses. The fragmentation generally includes three series of ions, as shown for the trimer MOC-HA-HA-HA-ME in Fig. 3. Most abundant are the fragments a_1, a_2 and a_3, in the case of the lactic acid derivative of *m/z* 275, 203 and 131 (Fig. 4). The fragments b_1 and b_2 and c_1 and c_2 (*m/z* 103,

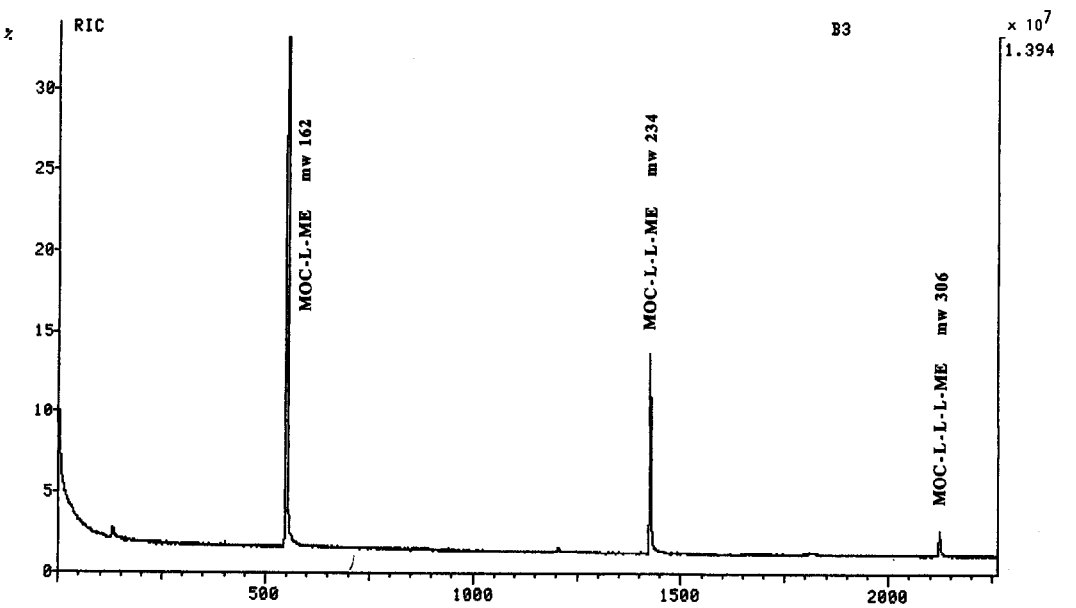

Fig. 2. Total ion chromatogram of the reaction products of D-lactic acid (L) with MCF and methanol. Abcissa, scan number (1000 scans in 12 min); left ordinate, relative intensity (%); right ordinate, counts.

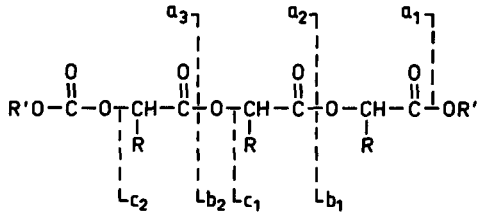

Fig. 3. Mass spectrometric fragmentation of "inter-esters" formed from 2-hydroxycarboxylic acids.

159, 175 and 231, respectively, for the D-lactic acid derivative) are less intense but very characteristic for all "inter-esters".

In the reaction with ECF, the other side-products are formed to a larger extent in addition to the "inter-esters", *i.e.*, the lactides and the substances postulated to be "inter-esters" with an underivatized OH group, as shown for D-lactic acid in Fig. 5. In the mass spectra of the lactides of the different 2-HAs the ion $[M+1]^+$ predominates, accompanied by $[M+29]^+$ and $[M+41]^+$. The mass spectra of the "inter-esters" with an underivatized OH group exhibit the same regularities as the alkoxycarbonyl "inter-esters". However, additionally an ion occurs corresponding to the loss of one 2-HA unit of the oligomer.

On reaction of 2-HAs with ECF and methanol instead of ethanol, both methyl and ethyl ester structures are formed. The major products are the methyl esters (Fig. 6). From this finding it can be concluded that the carboxylic group primarily reacts directly with the alcohol and not with alkyl chloroformate to form mixed anhydrides, which then undergo decarboxylation, as has been reported in connection with fatty acids and amino acids [4,5]. In the reaction of the 2-HAs with MCF and ECF, mixed anhydrides are not observed as side-products.

The described side-products are formed from all the 2-HAs studied except 2-hydroxyisobutyric acid. For this acid "inter-ester" formation is probably hindered for steric reasons. Likewise, "inter-esters" have not been observed in the reactions between MCF or ECF and hydroxycarboxylic acids other than 2-HAs, *e.g.*, D,L-3-hydroxybutyric acid. In this instance the main reaction product is the methyl and ethyl ester, respectively, with the OH group left underivatized.

When MCF and ECF are to be applied as deriv-

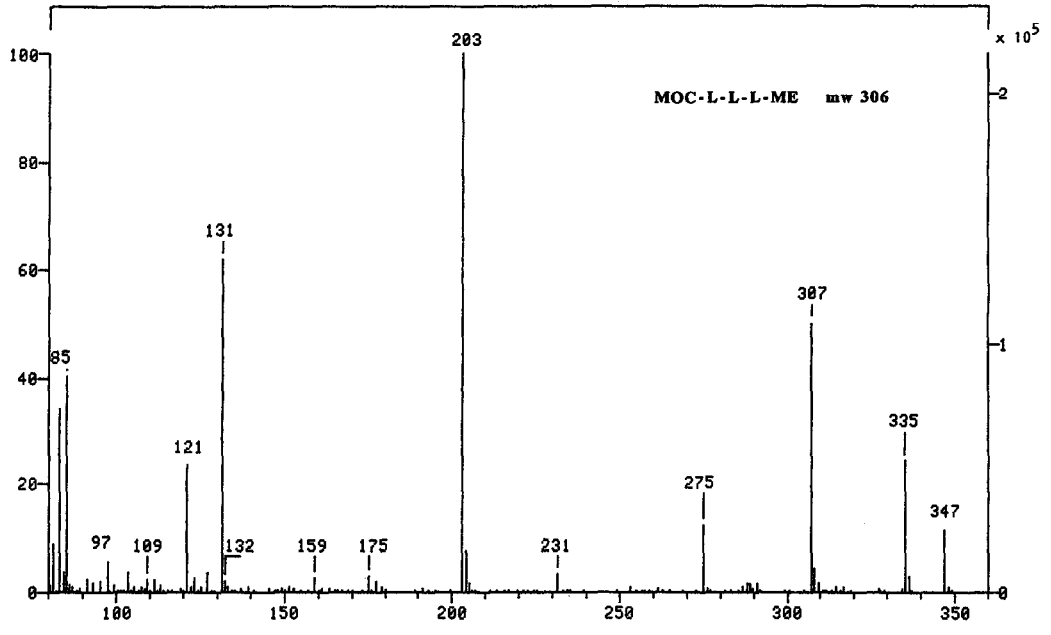

Fig. 4. CI mass spectrum of the D-lactic acid "inter-ester" trimer. Abcissa, *m/z*; left ordinate, relative intensity (%); right ordinate, counts.

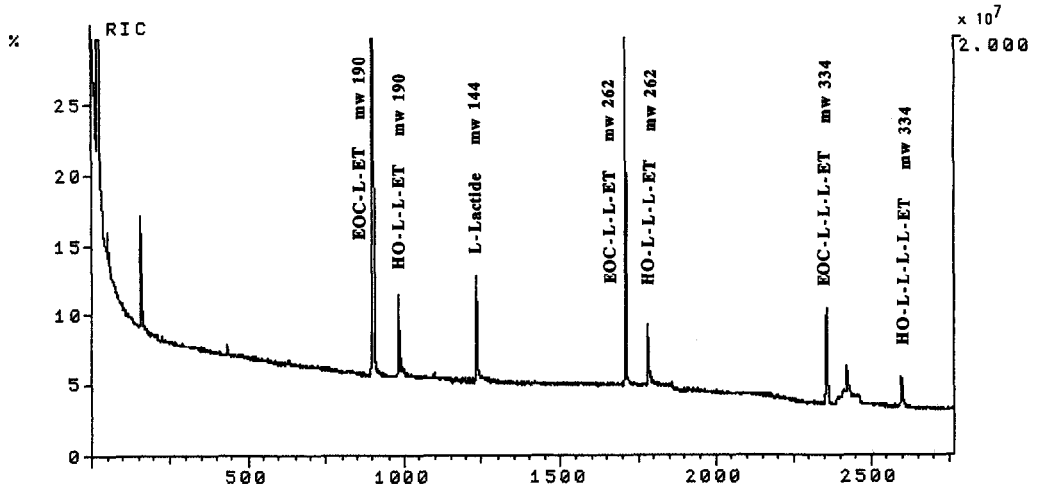

Fig. 5. Total ion chromatogram of the reaction products of D-lactic acid with ECF and ethanol. Abcissa, scan number (1000 scans in 12 min); left ordinate, relative intensity (%),; right ordinate, counts.

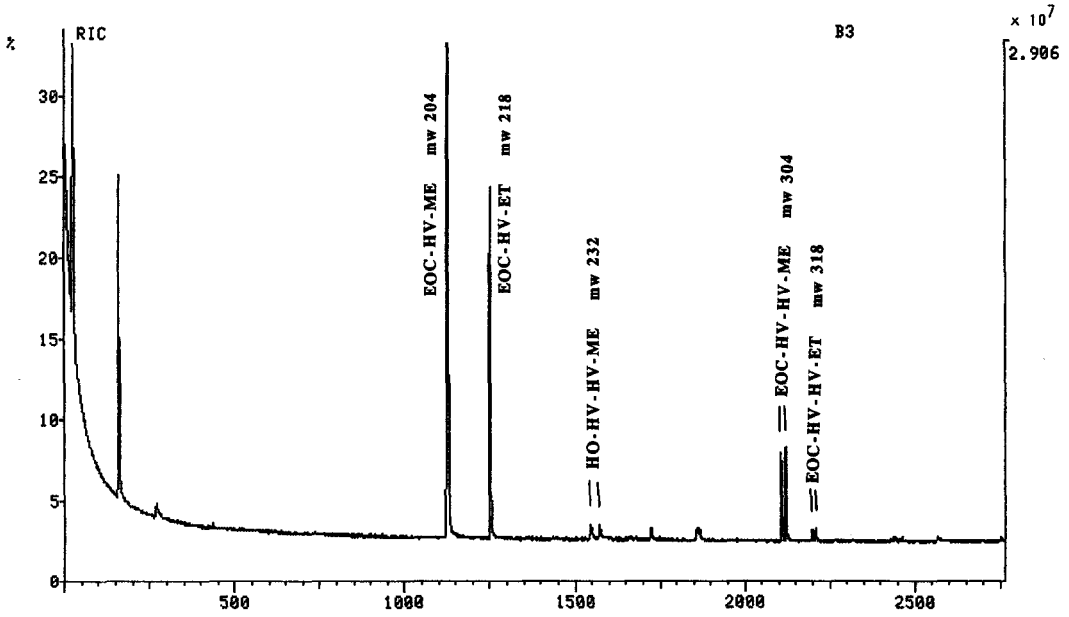

Fig. 6. Total ion chromatogram of the reaction products of D,L-2-hydroxyvaleric acid with ECF and methanol. Abcissa, scan number (1000 scans in 12 min); left ordinate, relative intensity (%); right ordinate, counts.

atization agents to 2-HAs, the ratios of aqueous solution, acetonitrile and alcohol have to be optimized in order to reduce side-product formation. In this study the reaction conditions were chosen with the aim of producing high yields of side-products to facilitate their identification. Complete elimination of side-products does not appear to be possible.

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