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ABSOLUTE CONFIGURATION OF 3-HYDROXYADIPIC ACID IN HUMAN URINE

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SUMMARY

A method involving derivatization and combined gas chromatography—mass spectrometry has been developed to separate the enantiomers of 3-hydroxyadipic acid. By combining this method with asymmetric synthesis of the same acid, it has been shown that 3-hydroxyadipic acid excreted in urine consists of at least 95% of the L-enantiomer. This finding supports the hypothesis that dicarboxylic acids are degraded by ordinary β-oxidation, and indicates that adipic acid may be converted into succinic acid.

INTRODUCTION

The advent of gas chromatography—mass spectrometry (GC—MS) instrumentation has made possible detailed analysis and structure elucidation of urinary organic acids, and this has resulted in the discovery of a variety of "new" metabolic disorders during the last two decades [1]. The urinary excretion of adipic and suberic acid during ketosis has previously been described [2]. Furthermore, increased urinary excretion of the two acids has been observed following the ingestion of longer dicarboxylic [3] and monocarboxylic

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[4, 5] acids. In ketosis, adipic acid and suberic acid may be formed from endogenous long-chain fatty acids, conceivably by tandem ω-oxidation—β-oxidation processes [4, 6].

In addition to adipic and suberic acid, the corresponding 3-hydroxylated acids have been detected in the urine of ketotic patients [7], and of volunteers ingesting dicarboxylic acids [8]. This indicates that 3-hydroxydicarboxylic acids may be intermediates in the degradation of dicarboxylic acids and, furthermore, suggests that the degradation involves an ordinary β-oxidation. If this were the case, the intermediate 3-hydroxylated diacids should be formed in the L-configuration. In order to shed more light on the metabolic degradation of dicarboxylic acids it would therefore be useful to ascertain the configuration of the corresponding 3-hydroxylated metabolites. The present paper describes our successful efforts toward determining the configuration of urinary 3-hydroxyadipic acid (*1*). Essentially, the method employed consists of chiral derivatization and gas chromatographic analysis on an achiral column [9].

EXPERIMENTAL

Chemicals

All of the chemicals used were commercial products of high purity supplied by Fluka (Buchs, Switzerland).

Synthesis of reference compounds

7-Oxabicyclo[4,1,0]hept-3-ene (*2*). A solution of *m*-chloroperbenzoic acid (40 mmol) in chloroform (200 ml) was added dropwise to 1,4-cyclohexadiene (40 mmol) dissolved in chloroform (75 ml). The reaction mixture was stirred at ambient temperature until the starch—iodine test was negative. The mixture was washed (aqueous sodium bicarbonate) and dried (sodium sulphate). The solvent was removed under reduced pressure, and the residue was distilled through a Vigreux column to give 2.42 g (63%) of *2* (lit. [10]: b.p. 72°C at 70 mmHg). The ^{1}H- and ^{13}C-NMR (nuclear magnetic resonance) spectra were in accordance with the literature [10, 11]. IR (infrared) (cm^{-1}): 2990 (s), 2890 (s), 2810 (w), 1480 (w), 1420 (s), 1215 (s), 1010 (m).

3-Cyclohexenol (*3*). A solution of *2* (13.6 mmol) in dry diethyl ether (75 ml) was added to a slurry of lithium aluminium hydride (10 mmol) in dry diethyl ether (50 ml). After stirring at ambient temperature for 5 h, water (5 ml) was added. Water and aluminium compounds were trapped by adding magnesium sulphate. The solid salts were filtered and washed with diethyl ether. The diethyl ether was then removed from the combined filtrates under reduced pressure, and the residue was distilled to give 1.05 g (85%) of *3* (lit. [12]: b.p. 90—94°C at 50 mmHg). IR and ^{1}H-NMR spectra were in accordance with the literature [12]. ^{13}C-NMR ($C^{2}H_{3}O^{2}H$): δ 127.5, 125.2, 67.7, 35.1, 31.9, 25.0. MS [*m/z*, relative abundance (%)]: 98 (2), 97 (2), 83 (7), 80 (100), 79 (24), 70 (8), 69 (10).

3-Cyclohexenol, O-acetyl-D-mandelic ester (*4a*). O-Acetyl-D-mandelic acid was prepared as described in the literature [13]. The corresponding acid chloride was prepared by adding freshly distilled thionyl chloride (100 mmol)

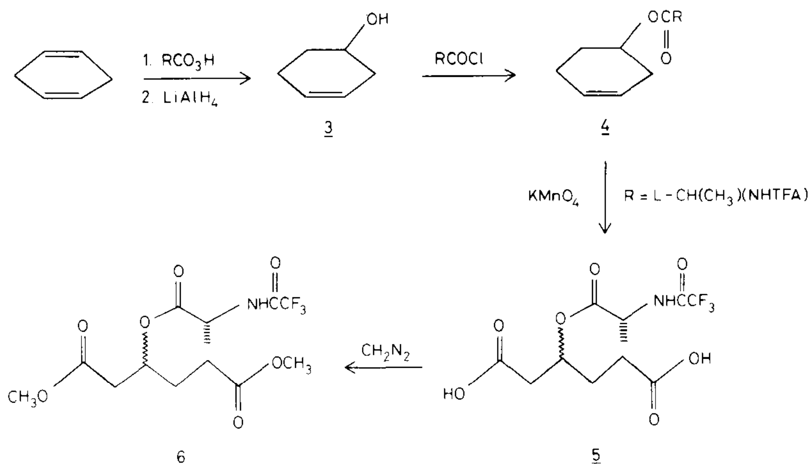
to a solution of O-acetyl-D-mandelic acid (25 mmol) in methylene chloride (10 ml). Unreacted thionyl chloride was evaporated in a stream of dry nitrogen, and the residue was dissolved in methylene chloride (5 ml). 3-Cyclohexenol (10 mmol) was added to this solution, and this mixture was occasionally shaken over a period of four days. When the reaction was complete, water (10 ml) was added. The water phase was made slightly basic by adding aqueous sodium bicarbonate and extracted with diethyl ether (3 × 10 ml). The combined extracts were dried (sodium sulphate) and worked up in the usual way to give a quantitative yield of *4a* which was used for gas—liquid chromatographic (GLC) analysis without further purification.

3-Cyclohexenol, N-trifluoroacetyl-L-phenylalanyl ester (*4b*). N-TFA-L-phenylalanine was made by carefully adding trifluoroacetic anhydride (50 mmol) to L-phenylalanine (10 mmol) which was cooled on an ice-bath. After the amino acid had dissolved, unreacted anhydride was removed by a stream of dry nitrogen. Thionyl chloride (100 mmol) was added, and after 8 h unreacted thionyl chloride was removed as described for compound *4a*. Alcohol *3* was esterified to give *4b* which was worked up as described for ester *4a*.

3-Cyclohexenol, N-TFA-L-prolyl ester (*4c*). N-TFA-L-prolyl chloride was prepared according to literature procedures [14], and the esterification and isolation were performed as described for ester *4a*.

3-Cyclohexenol, N-TFA-L-alanyl ester (*4d*). N-TFA-L-alanyl chloride was prepared according to standard literature procedures [15]. The esterification of *3* and the isolation of *4d* were performed as described for ester *4a*. ^{1}H-NMR (C^2HCl_3): δ 5.6 (2H, m), 4.1 (1H, s), 4.0–3.6 (2H, m), 2.6—1.3 (9H, m). ^{13}C-NMR (C^2HCl_3): δ 171.2, 126.9, 126.8, 123.3, 123.1, 72.1, 72.0, 30.6, 30.5, 27.1, 27.0, 23.0, 22.9, 17.9, 17.8.

Triester 6. This compound was prepared in two steps (Scheme 1). Acid *5* was made by oxidation of *4d* with potassium permanganate according to a literature procedure [16]. Ester *4d* (2.5 mmol) and triethylbenzylammonium chloride were dissolved in benzene (15 ml) and stirred with a solution of potassium permanganate (11 mmol) in water (30 ml) for 2 h. The reaction was quenched by adding sodium bisulphite and hydrazine sulphate, and was



Scheme 1.

worked up in the usual way. The residue (0.72 g, 80%) was essentially pure *5*. ^{1}H-NMR ($C^2H_3O^2H$): δ 5.4 (1H, m), 4.6 (1H, m), 3.6 (1H, m), 2.8—1.6 (6H, m), 1.5 (3H, d). ^{13}C-NMR (C^2HCl_3): δ 175.6, 175.5, 172.6, 171.0, 170.8, 71.6, 49.2, 38.7, 29.7, 29.2, 29.1, 16.8. IR (cm^{-1}): 2970 (s), 2850 (s), 1730 (s), 1700 (s), 1680 (s), 1570 (s).

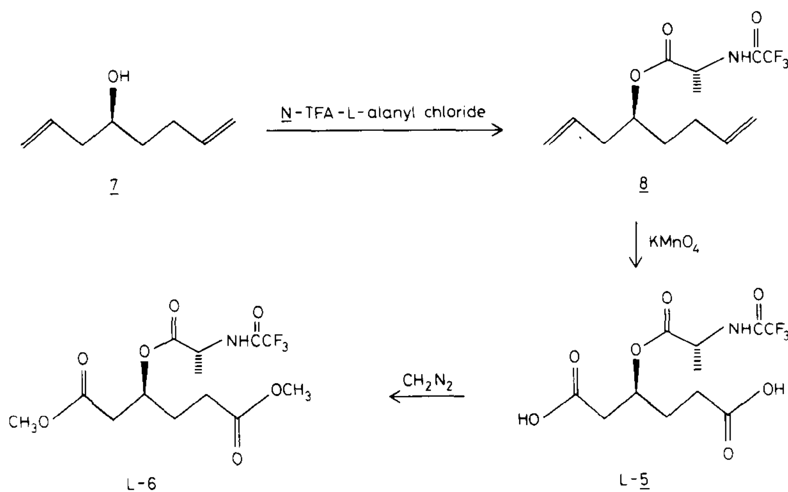
Triester *6* was then obtained in quantitative yield by treating *5* with an ethereal solution of diazomethane. ^{1}H-NMR ($C^2H_3O^2H$): δ 5.2 (1H, m), 4.6 (1H, m), 3.3 (6H, s), 2.8—1.6 (6H, m), 1.5 (3H, d). ^{13}C-NMR ($C^2H_3O^2H$): δ 172.3, 72.4, 39.6, 29.4, 29.1, 29.0, 16.7. IR (cm^{-1}): 3040 (m), 2980 (s), 2880 (m), 1740 (s), 1685 (s), 1550 (s). MS [*m/z*, relative abundance (%)] : 174 (7), 173 (16), 159 (16), 141 (47), 140 (73), 114 (12), 113 (45), 109 (18), 101 (17), 85 (14), 81 (19), 71 (100).

L-1,7-Octadien-4-ol, N-TFA-L-alanyl ester (*8*). L-1,7-Octadien-4-ol (*7*) (0.20 g, 1.6 mmol), prepared from L-malic acid as outlined by Bartlett [17], was esterified by N-TFA-L-alanyl chloride (3.25 g, 16 mmol) as described for compound *4d*. The product was obtained in quantitative yield when worked up in the usual way. ^{1}H-NMR ($C^2H_3O^2H$): 5.4 (2H, m), 5.0 (5H, m), 4.5 (1H, m), 2.6 1.1 (9H, m). ^{13}C-NMR ($C^2H_3O^2H$): 172.8, 136.2, 134.0, 118.3, 115.7, 76.1, 39.8, 33.0, 27.4, 16.7. IR (cm^{-1}): 3270 (m), 3030 (m), 2880 (s), 2800 (s), 1715 (s), 1635 (m), 1540 (m), 1345 (m), 1150 (s), 900 (m). MS [*m/z*, relative abundance (%)] : 168 (18), 141 (8), 140 (100), 109 (6), 108 (5), 93 (14), 92 (4).

Triester L-6. Ester *8* (0.100 g, 0.35 mmol) was oxidized as described for compound *4d* to give diacid L-*5* (0.099 g, 88%) which was converted to triester L-*6* in quantitative yield by treatment with diazomethane (Scheme 2). The spectral data of L-*6* were identical to those of the 1:1 diastereoisomeric mixture of *6*, except for the expected intensity differences in the ^{13}C-NMR spectra (see Fig. 2).

Preparation of urine extracts

Urine was collected quantitatively for 12 h from a healthy human given



Scheme 2.

dodecanedioic acid (20 mmol) perorally. An aliquot (100 ml) of the urine was acidified with 1 *M* hydrochloric acid to pH 1 and saturated with sodium chloride. This solution was extracted with ethyl acetate (3 × 100 ml) at 4°C, dried (sodium sulphate), and evaporated under reduced pressure, until a brown viscous oil was obtained. N-TFA-L-alanyl chloride (10 mmol) was added, and this solution was occasionally shaken for three days. Unreacted N-TFA-L-alanyl chloride was hydrolysed by adding water (25 ml) and the solution was extracted with ether (3 × 25 ml), dried (sodium sulphate) and evaporated under reduced pressure. The residue was dissolved in methanol and treated with an ethereal solution of diazomethane prior to GC—MS analysis.

Capillary GLC

A Hewlett-Packard HP-5880A gas chromatograph, equipped with a flame-ionization detector and a fused-silica column (25 m × 0.25 mm I.D.) coated with CP-Sil 19 CB (Chrompack) or OV-101 (Hewlett-Packard) was used for analysis of the reference compounds. Injections were made on a split injector with a split ratio of 1:30. The carrier gas (hydrogen) had a linear gas flow-rate of 45 cm/sec (at room temperature). The injector port and detector temperatures were 230°C and 270°C, respectively, whereas the oven temperature was optimized for each sample.

Gas chromatography—mass spectrometry

The measurements were performed with a VG Analytical MicroMass 7070H double-focusing mass spectrometer equipped with a VG Data System 2050 (PDP 8a computer), a Hewlett-Packard 5710 gas chromatograph, and a digital multiple ion detector unit controlled by the data system. The gas chromatograph was equipped with a fused-silica capillary column (25 m × 0.25 mm I.D.) coated with Carbowax 20M (Hewlett-Packard). Injection port temperature was 250°C, and the helium flow-rate was 2 ml/min. The interface was a direct jet inlet, heated to 250°C. Ionizing and accelerating potentials were 70 eV and 4 kV (standard, otherwise controlled by the multiple ion detector unit), respectively, and the temperature in the ion source was 220°C.

NMR spectroscopy

The ^{1}H-NMR spectra were obtained on a Jeol PMX 60 si spectrometer (60 MHz) at 35°C. The samples were 1—5% by weight in $C^{2}HCl_3$ or CCl_4 with tetramethylsilane as internal standard. The ^{13}C-NMR spectra were recorded on a Jeol FX 90 Q instrument (22.50 MHz) at 29°C. The samples, with tetramethylsilane as internal reference, were 5—10% by weight in $C^{2}HCl_3$ or $C^{2}H_3O^{2}H$ which provided the deuterium signal for the NMR field lock. The spectra were run with a spectral width of 5000 Hz, a pulse width of 5.5 μsec (45°) and a pulse repetition time of 5 sec. The computer operation conditions gave a digital resolution of 0.3 Hz. The C—H decoupling was made by a broad band decoupling pulse at 89.55 MHz.

IR spectroscopy

The infrared spectra were obtained on a Shimadzu IR-420 infrared spectrophotometer either as liquid film or potassium bromide tablet.

RESULTS AND DISCUSSION

In order to be able to determine the configuration of 3-hydroxyadipic acid (*1*) formed by metabolic hydroxylation of adipic acid, it is necessary to develop a method which allows enantiomeric separation of *1* or a derivative of *1*. A common method employed to achieve such separation of racemic alcohols on an analytical scale is to make diastereomeric esters by reacting the alcohols with a chiral acid chloride. Among the best and most convenient reagents for this purpose are N-TFA amino acid chlorides which are readily available and usually give good enantiomeric resolution when the resulting diastereoisomeric mixtures are analysed by GLC [18]. To see if this scheme could be adopted for the analysis of racemic and optically active *1* we first performed exploratory separation experiments with various N-TFA amino acid esters (*4*) of 3-cyclohexenol, an intermediate in the synthesis of racemic *1* (Scheme 1). GLC analysis of the esters on an OV-101 column revealed that the enantiomeric separation varied with the amino acid moiety attached to *3*. Thus, only modest separation was obtained with aromatic esters whereas aliphatic esters showed higher separation ability (Table I). The N-TFA-L-alanyl ester derivative, *4d*, proved to be the best and was chosen for subsequent synthetic transformations.

TABLE I

ENANTIOMERIC SEPARATION OF SOME DERIVATIVES OF 3-CYCLOHEXENOL ON AN OV-101 CAPILLARY COLUMN

Derivative	*R* factor
O-Acetyl-D-mandelic ester	0.6
N-TFA-L-phenylalanyl ester	0.8
N-TFA-L-prolyl ester	1.0
N-TFA-L-alanyl ester	1.2

Permanganate oxidation of *4d* gave a 1:1 diastereoisomeric mixture of the N-TFA-L-alanyl ester *5* of 3-hydroxyadipic acid which was transformed into a diastereoisomeric mixture of the corresponding triester *6* by treatment with diazomethane (Scheme 1). Due to higher polarity compound *6* showed lower enantiomeric separation than ester *4d*; the resolution factor was as low as 0.8, when the GLC analysis was performed on an OV-101 column. However, by increasing the polarity of the column the resolution improved so significantly that baseline separation of the diastereoisomers was achieved (Table II and Fig. 1a).

The diastereoisomeric mixture of *6* can also be analysed by ^{13}C-NMR spectroscopy. The basis for this is that diastereoisomers usually show a pair of signals, one signal for each diastereoisomer, for one or several of the carbon atoms in the molecule. Triester *6* gives rise to three such pairs (Fig. 2a). Two of them, situated at approximately 172 and 176 ppm, are due to the carbon atoms in two of the carboxyl groups and have shift differences of 0.2(2) and 0.1(3) ppm, respectively. The third pair, at approximately 30 ppm, has a shift difference of 0.0(7) ppm and is caused by one of the methylene groups

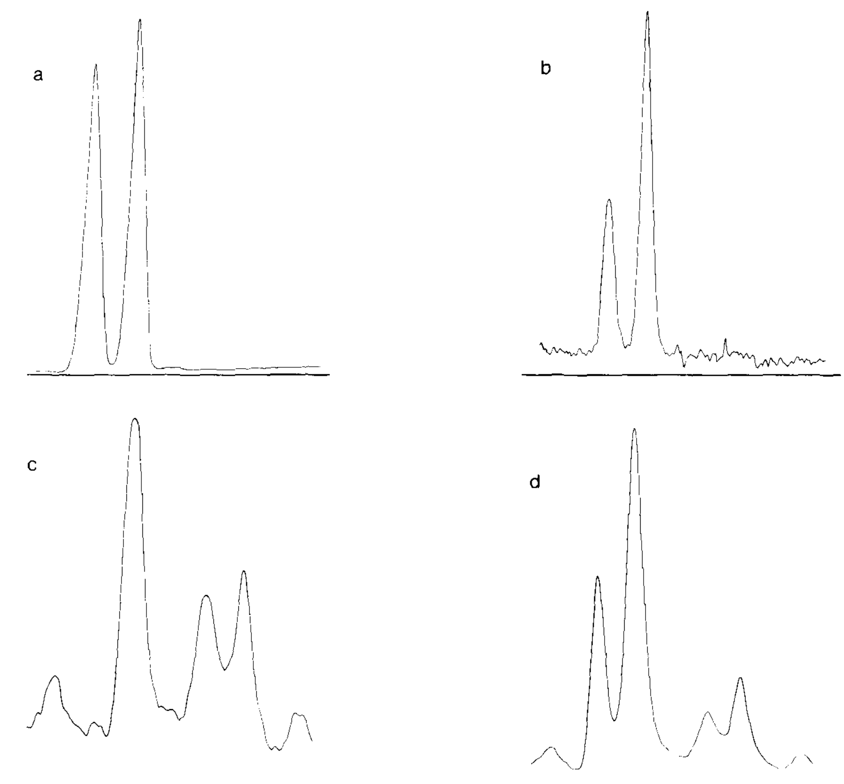


Fig. 1. Multiple ion detection gas chromatograms at m/z = 109 of (a) a 1:1 mixture of D-*6* and L-*6*, (b) a 35:65 mixture of D-*6* and L-*6*, (c) parts of the gas chromatogram of a derivatized ethyl acetate urinary extract, (d) parts of the gas chromatogram of a derivatized ethyl acetate urinary extract to which has been added some of the 1:1 diastereoisomeric mixture of *6*.

TABLE II

ENANTIOMERIC SEPARATION OF 3-HYDROXYADIPIC ACID, N-TFA-L-ALANYL ESTER AS DIMETHYL ESTER ON VARIOUS GLC COLUMNS

Column	*R* factor
OV-101	0.8
CP-Sil 19 CB	1.2
Carbowax 20M	1.5

in the adipic acid moiety. Within each pair the integrals were equal, so there is no significant difference in relaxation times and nuclear Overhauser effects for diastereoisomeric carbons. Thus, two independent methods are available for the analysis of *1*.

To determine the configuration of urinary 3-hydroxyadipic acid it is necessary to synthesize optically active *1* with known configuration. This was done by permanganate oxidation of *8*, prepared from L-1,7-octadien-4-ol (*7*) and N-TFA-L-alanyl chloride (Scheme 2). Optically active *7*, with the L-enantiomer predominating, was synthesized from L-malic acid [17]. The product thus

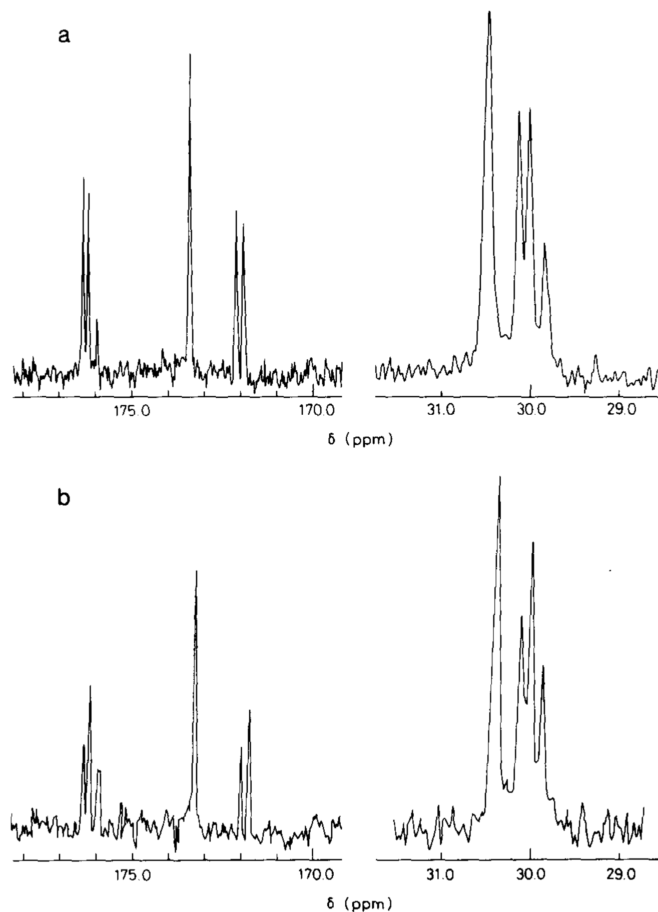


Fig. 2. Parts of the ^{13}C-NMR spectrum of (a) a 1:1 diastereoisomeric mixture of *5*, (b) a 34:66 diastereoisomeric mixture of D-*5* and L-*5*.

obtained, L-*5*, was converted to triester L-*6* in the usual way and analysed by GLC. This analysis revealed that L-*6* was contaminated with 35% of D-*6* and, furthermore, that L-*6* has the longer retention time of the two diastereoisomers under the conditions employed (Fig. 1b). This result was confirmed by a ^{13}C-NMR investigation of the unesterified mixture of *5*; on average the separable peaks due to the two diastereoisomers showed a relative intensity corresponding to a 66:34 mixture of L-*5* and D-*5* (Fig. 2b).

The formation of the D-enantiomer of *5* may have occurred during the preparation of *7* or during its subsequent esterification and oxidation (Scheme 2). In order to clarify this a lanthanide shift experiment was performed on *7*. By addition of a chiral shift reagent, tris[*d*,*d*-dicamphylmethanato]europium(III), to the alcohol the allylic hydrogen atoms in the dienol moiety of the D-enantiomer of *7* are completely separated from the corresponding hydrogen atoms due to L-*7* [18]. By comparing the integrals of the separated peaks it was established that *7* was a 37:63 mixture of the D- and L-enantiomers, respectively. Thus, racemization took place prior to the esterification and oxidation steps.

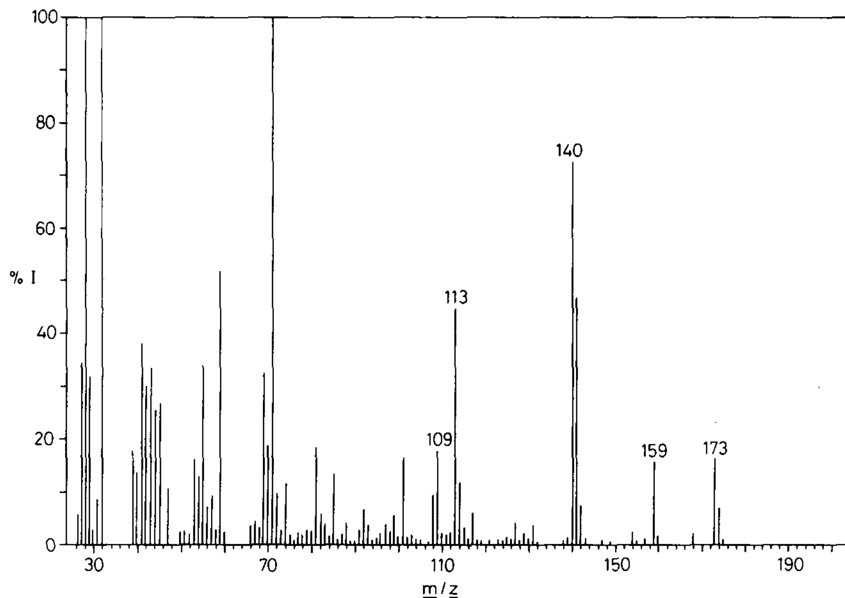


Fig. 3. The electron-impact mass spectrum of a 1:1 diastereoisomeric mixture of *6*.

The mass spectrum of *6* (Fig. 3) showed extensive fragmentation and no molecular ion; furthermore, there was no difference between the mass spectra of the diastereoisomers. The fragment at m/z = 173 is probably due to cleavage of the C—O ester bond in position 3 of the adipic acid moiety. Analogous fragmentation is known from simple *n*-alkyl-N-TFA-L-alanyl esters [19]. The dominant peak at m/z = 140 is characteristic for N-TFA-alanyl esters [20] and is assigned to α-cleavage between the carbonyl group and the α-carbon atom in the alanyl group. Another typical fragmentation process from such esters is α-cleavage on the other side of the carbonyl group, which in this case results in a weak peak with m/z = 168. N-TFA-alanyl esters always have a rather strong peak at m/z = 141, but in the present case this peak may also be due to a loss of methanol from the fragment at m/z = 173. The peak at m/z = 113 originates partly from m/z = 141, by C=O extrusion, and partly from m/z = 173, by elimination of methyl formate (confirmed by metastable daughter-ion scanning from m/z = 173 and 141).

One problem which frequently arises when biological samples are analysed by GC—MS, is that the compound of interest is masked by one or more co-eluting substances. This problem can be avoided by using high-resolution gas chromatography which normally gives well resolved and narrow peaks. Unfortunately, this often generates new problems because the peaks may be so narrow that it is impossible to obtain a full mass spectrum on magnetic scanning instruments. To circumvent these problems magnetic scanning can be replaced by rapid switching of the accelerating and electrostatic analyser voltages between preset values which correspond to characteristic fragments in the mass spectrum of the compound under consideration. This method is extensively used in quantitative measurements (when the number of masses is small), but by increasing the number of m/z values the selectivity can be almost as great as

in the full scanning mode. The main disadvantage with the multiple ion detection method is that the mass spectrum of the compound under consideration must be known, but in the present case this is no obstacle since the necessary reference compounds can be prepared both optically active and as racemates.

The determination of the configuration of metabolic 3-hydroxyadipic acid starts with derivatization of an ethyl acetate extract from a urine sample. Treatment with N-TFA-L-alanyl chloride and diazomethane converts *1* into the same derivatives as the synthetic standards, although some of the acid is lost by lactonization [8]. The sample is now ready for multiple ion detection analysis which was performed on eight of the fragment ions from the mass spectrum of the synthetic standard (Fig. 4). Comparison of the mass selective chromatograms showed that one peak matched all eight ions, the retention time of which was the same as that of the L-enantiomer of the synthetic compound (Fig. 1b and c). This finding was further substantiated by adding some of the 1:1 diastereoisomeric mixture of *6* to the urine sample before injection; this gave an increased amount of the L-enantiomer, together with a new peak with the same retention time as the synthetic D-form (Fig. 1d).

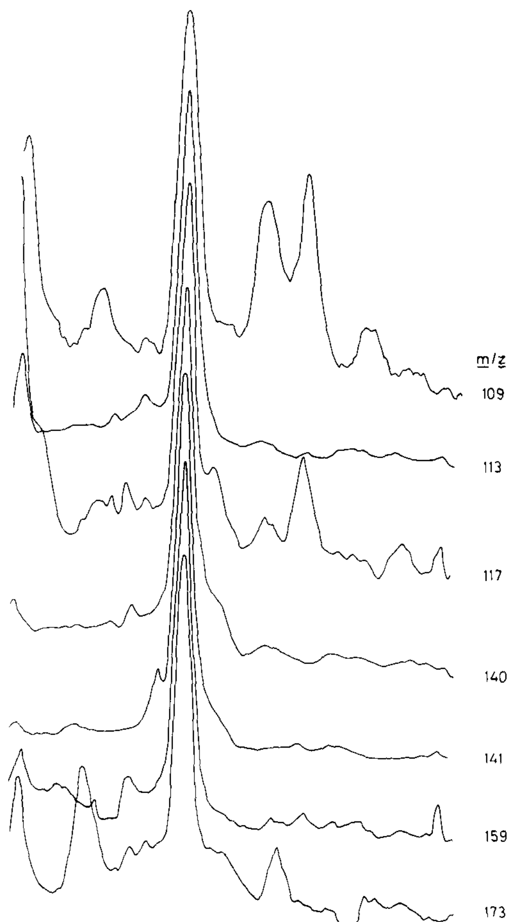


Fig. 4. GC—MS multiple ion detection chromatogram of an ethyl acetate urine extract after derivatization with N-TFA-L-alanyl chloride and diazomethane. The ions selected are the most characteristic ones in the mass spectrum of *6*.

CONCLUDING REMARKS

It has previously been demonstrated that administration of higher (C_8–C_{12}) ^{14}C-labelled dicarboxylic acids to ketotic rats gave a decrease in circulating ketone bodies and an increase in [^{14}C]glucose [21]. Administration of adipic acid gave the same antiketogenic effect, together with a rise in blood glucose and succinic acid [3]. These and other studies have indicated that adipic acid can be further oxidized in the body [22, 23] probably by β-oxidation [8, 24]. Formation of succinic acid, by β-oxidation of adipic acid, may lead to an increased glucose production and be of physiological importance in the regulation of ketosis. Our finding of L-3-hydroxyadipic acid in urine supports the hypothesis that dicarboxylic acids are degraded by ordinary β-oxidation, and indicates that adipic acid may be converted into succinic acid.

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