Aliphatic medium chain tricarboxylic acids in rat urine

Birgitta Eliasson, Sven Lindstedt, and Göran Steen

Department of Clinical Chemistry, University of Gothenburg, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden

Abstract Three aliphatic tricarboxylic acids have been found in rat urine. They have been identified as 6-carboxy-5-undecenedioic acid, 6-carboxy-5-dodecenedioic acid, and 6-carboxy-5-tridecenedioic acid. The carbon skeleton structure was determined by mass spectra of the hydrogenated methyl esters. The double bond position was determined after osmium tetroxide oxidation followed by trifluoroacetylation and mass spectrometry and by infrared spectrometry. The compounds were present in the urine when the rats were fed on pellets but disappeared when they received sucrose and water. The acids were not present in the pellets, and a metabolic relation to compounds of longer chain length, possibly mycolic acids, is likely.

Supplementary key words 6-carboxy-5-undecenedioic acid · 6-carboxy-5-dodecenedioic acid · 6-carboxy-5-tridecenedioic acid · metabolites · mycolic acids · mass spectrometry

Studies of aliphatic acids in urine by gas–liquid chromatography and mass spectroscopy have resulted in the identification of some unusual dicarboxylic acids, e.g., acids containing cyclopropane rings (1) or acetylenic bonds (2). These acids presumably arise from parent acids of longer chain length by a combination of ω-oxidation and β-oxidation. We now report the identification of homologous tricarboxylic acids in rat urine.

MATERIALS

Diethyl ether and petroleum ether (bp 40–60°C) of analytical grade, platinum oxide, and osmium tetroxide were from BDH Chemicals, Poole, England. *N*-Nitrosotoluene-4-sulfomethylamide was from Fluka AG, Buchs, Switzerland. Silicic acid (100 mesh) was from Mallinckrodt Chemical Works, St Louis, Mo. To remove fine particles, the silicic acid was suspended in water and allowed to sediment for 10 min before decanting the supernatant fluid. This procedure was repeated three times, and the silicic acid was then dried at 120°C for 24 hr. Silver nitrate was from E. Merck AG, Darmstadt, West Germany.

Column packing for gas–liquid chromatography, 3% OV-17 on 100–120 mesh Gas-Chrom P, was from Applied Science Laboratories, State College, Pa. *N,O*-Bis-(trimethylsilyl)-trifluoroacetamide and trifluoroacetic anhydride were from Pierce Chemical Co., Rockford, Ill. Hydroxylamine hydrochloride was from Sigma Chemical Co., St Louis, Mo.

Albino rats of the Wistar strain (200–400 g) were fed pellets for rats and mice obtained from Astra-Ewos, Södertälje, Sweden. According to the producer, these pellets contain fish protein, roasted soya meal, meat meal, cereals, animal fat, soya lecithin, minerals, vitamins, and trace elements.

METHODS

Urine collection

Rats were kept in stainless steel metabolic cages. The urine was collected in 24-hr portions in flasks containing a few drops of 6 M hydrochloric acid, and then frozen to −20°C.

Analytical procedures

Extraction and esterification. The acidity of the urine was adjusted to pH 1. The urine was extracted three times with equal volumes of diethyl ether. Combined extracts were concentrated under reduced pressure, dried over sodium sulfate (5 g/100 ml) and treated with diazomethane in ethereal solution (3). The treatment was continued until no more reaction was noted, usually less than 5 min.

Column chromatography. The adsorbent was silicic acid or silicic acid–silver nitrate. The latter was prepared by mixing 90 g of silicic acid with 10 g of silver nitrate in 200 ml of water and drying at 120°C for 24 hr. The columns were packed in diethyl ether–petroleum ether 20:80 (v/v) and samples were applied in the same solvent mixture. Elution was then performed with stepwise increases in the proportion of diethyl ether, or with a constant high proportion

TABLE 1. Preparation of aliphatic tricarboxylic acids (I, II, and III) by column chromatography

Column Designation	Type of Adsorbent and Amount	Column Dimensions (cm)	Sample Applied	Eluant:Proportion Diethyl Ether (ml/100 ml) and Volume	Fraction Volume (ml) and Number of Fractions	Fractions with Tricarboxylic Acids
A	Silicic acid 50 g	32 × 2	4.69 g of urine extract	20,25,30,40, and 50 500 ml of each eluant	166 ml 15	7–9,[a] 483 mg,[b] I–III,[c] 15%
B	Silicic acid 20 g	59 × 0.9	434 mg of A 7–9	20,22,24,26,28,30, and 32 200 ml of each eluant	20 ml 70	26–31, 31 mg, III 30% 32–43, 54 mg, II 15% 56–68, 32 mg, I 80%
C	Silicic acid–silver nitrate 6 g	21 × 0.9	28 mg of B 26–31	50 60 ml	1 ml 60	24–25, 1.5 mg, III, > 97% 26–30, 9.0 mg, III, 70%
D	Silicic acid–silver nitrate 9 g	30 × 0.9	9 mg of C 26–30	50 90 ml	1 ml 90	36–42, 3 mg, III, > 97% 43–45, 2.8 mg, III, 60–70%
E	Silicic acid 14 g	48 × 0.9	11 mg of B 32–43	22,23,24,25,26,27, and 28 150 ml of each eluant	15 ml 70	42–45, 1.1 mg, II, 97%
F	Silicic acid–silver nitrate 9 g	30 × 0.9	7 mg of B 56–68	50 90 ml	1 ml 90	25–32, 1.0 mg, I, > 97% 33–39, 3.0 mg, I, 90%

[a] Fraction numbers.
[b] Dry weight.
[c] Compound.
[d] Area of I–III/total peak area, by gas–liquid chromatography.

of diethyl ether. In the latter case, the eluant was collected in smaller fractions. Fractions were first analyzed by gas–liquid chromatography and then appropriately combined and weighed. Details are summarized in **Table 1.**

Gas–liquid chromatography. The gas chromatograph was a model 5750 (Hewlett-Packard Instruments, Avondale, Pa.), equipped with a flame-ionization detector. The stationary phase, 3% OV-17 on a support of Gas-Chrom P, was packed into glass columns of 2 m × 4 mm (ID). The mobile phase was helium at a flow rate of 30 ml/min. For rapid analysis of fractions from chromatographic columns, the oven temperature was programmed from 150°C to 300°C at a rate of 8°C per min. For quantitative determinations, *n*-decanedioic acid was added as internal standard. No corrections were made for detector response ratios.

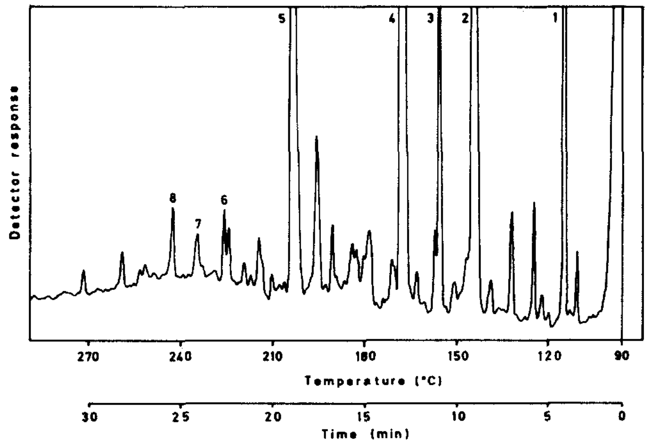


Fig. 1. Gas–liquid chromatography of a methylated diethyl ether extract of rat urine. The column packing was 3% OV-17, and the temperature was programmed from 90°C to 290°C by 6°/min. Peaks were identified as (*1*) glutaric acid, (2) heptanedioic acid, (*3*) octanedioic acid, (*4*) nonanedioic acid (*5*) p-hydroxyphenylpropionic acid. Peaks 6–8 correspond to compounds *I–III*, aliphatic tricarboxylic acids with 12–14 carbon atoms.

Mass spectrometry. Mass spectra were recorded with an LKB 9000 combined gas chromatograph–mass spectrometer (LKB-Produkter AB, Bromma, Sweden) with use of the same kind of column as for gas–liquid chromatography. The acceleration voltage was 3.5 kV, electron energy 70 eV, and ion-source temperature 270°C.

Infrared spectrometry. Infrared spectra were recorded on a Model 257 spectrometer (Perkin-Elmer Corp., Norwalk, Conn.) using 100-μl sodium chloride cuvettes. The sample, 0.5 mg, was dissolved in carbon tetrachloride.

Trimethylsilylation. The sample, 0.1–0.5 mg, was dissolved in 100 μl of *N,O*-bis (trimethylsilyl)trifluoroacetamide–acetonitrile 1:1 (v/v), and the solution was kept in the dark at room temperature for 30 min. Alternatively *N,O*-bis (d_9-trimethylsilyl)acetamide was used for silylation (Sharp and Dohme, West Germany).

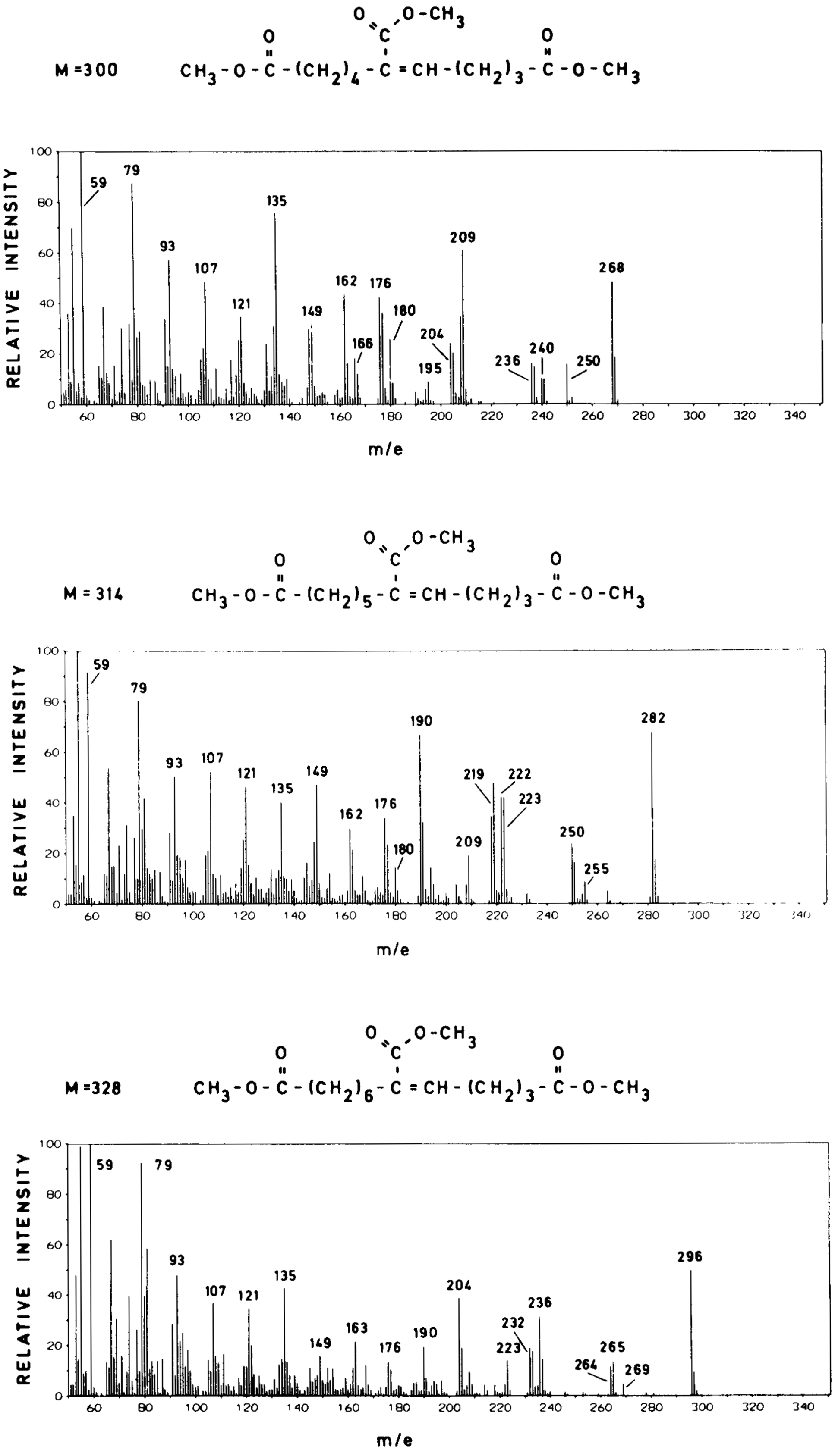


Fig. 2. Mass spectra of the methyl esters of compounds *I* (top spectrum), *II*, and *III*. For interpretation, see Table 2.

TABLE 2. Ions formed in mass spectra of methyl esters of compounds I, II and III (compare Fig. 2)

	Mass Number of Ion Observed (*m/e*)		
Formation of Fragment Ion	I (M = 300)	II (M = 314)	III (M = 328)
$M - CH_3O$[a]	269	283	297
$M - CH_3OH$	268	282	296
$M - CH_3OCO$	241	255	269
$M - [CH_3OCO + H]$	240	254	268
$M - CH_3O - CH_3OH$	237	251	265
$M - CH_3OH - CH_3OH$	236	250	264
$M - CH_3O - [CH_3OCO + H]$	209	223	237
$M - CH_3OH - [CH_3OCO + H]$	208	222	236
$M - CH_3O - [CH_3OCOCH_2 + H]$	195	209	223
$M - CH_3OH - [CH_3OCOCH_2 + H]$	194	208	222
$M - CH_3OCO - [CH_3OCO + H]$	181	195	209
$M - [CH_3OCO + H] - [CH_3OCO + H]$	180	194	208
$M - CH_3OCO - [CH_3OCOCH_2 + H]$	167	181	195
$M - [CH_3OCO + H] - [CH_3OCOCH_2 + H]$	166	180	194
$M - CH_3O - CH_3OH - CH_3OH$	205	219	233
$M - CH_3OH - CH_3OH - CH_3OH$	204	218	232
$M - CH_3O - CH_3OH - [CH_3OCO + H]$	177	191	205
$M - CH_3OH - CH_3OH - [CH_3OCO + H]$	176	190	204
$M - CH_3O - CH_3OH - [CH_3OCOCH_2 + H]$	163	177	191
$M - CH_3OH - CH_3OH - [CH_3OCOCH_2 + H]$	162	176	190
$M - CH_3O - [CH_3OCO + H] - [CH_3OCO + H]$	149	163	177
$M - CH_3OH - [CH_3OCO + H] - [CH_3OCO + H]$	148	162	176
$M - CH_3O - [CH_3OCO + H] - [CH_3OCOCH_2 + H]$	135	149	163
$M - CH_3OH - [CH_3OCO + H] - [CH_3OCOCH_2 + H]$	134	148	162

[a] Also containing isotopic species of $M - CH_3OH$.

Formation of trimethylsilylated oximes. The sample, 0.1–0.5 mg, was dissolved in 50 μl of hydroxylamine hydrochloride in pyridine (20 mg/ml solution), and kept at room temperature for 30 min. The sample was then trimethylsilylated as above.

Trifluoroacetylation. The sample, 0.1–0.5 mg, was dissolved in 100 μl of trifluoroacetic anhydride–acetonitrile 1:1 (v/v), and the solution was kept at room temperature for 1 hr.

Preparation of trideuterated methyl esters. Trideuterated methyl esters were prepared by alkaline hydrolysis (2) of 0.2 mg of methyl ester, followed by treatment of the acid with 100 μl of Tri-deuter 8 (Pierce Chem. Co.), for 30 min at 60°C.

Catalytic hydrogenation. Platinum oxide, 5 mg suspended in 0.5 ml of methanol, was reduced by flushing with hydrogen gas. The sample, 0.1–0.5 mg, dissolved in methanol was hydrogenated for 90 min at room temperature. The catalyst was removed by filtration and the solvent evaporated.

Osmium tetroxide oxidation. Treatment with osmium tetroxide and purification of products was done as previously described (2). The products were trimethylsilylated or trifluoroacetylated before gas–liquid chromatography–mass spectrometry.

RESULTS

Occurrence of tricarboxylic acids in rat urine

Fig. 1 shows a gas chromatogram of a methylated diethyl ether extract of rat urine. A large number of peaks are present. As judged from mass spectra, peaks 6 to 8 contained tricarboxylic acids of unusual structure and further chromatographic procedures were carried out to isolate these compounds, referred to as compounds *I* to *III*.

Isolation by column chromatography

Compounds *I* to *III* were isolated from 4.3 l of rat urine, which had been collected from six rats over a period of about two months. The diethyl ether extract, 4.69 g dry weight after methylation, was fractionated through a series of chromatographic

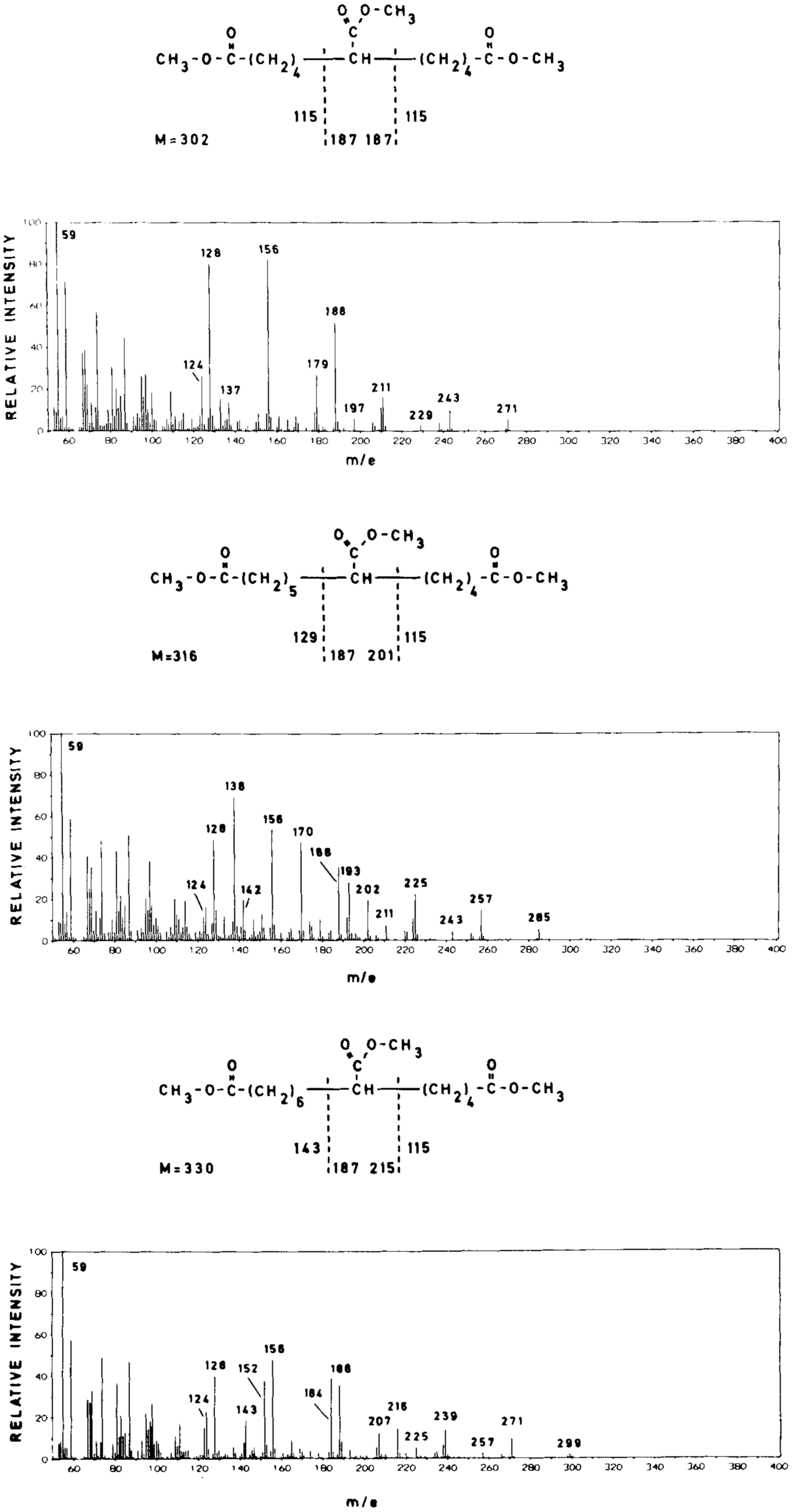


Fig. 3. Mass spectra of the methyl esters of compounds *I* (top spectrum), *II*, and *III* after catalytic hydrogenation. For interpretation, see Table 3.

TABLE 3. Ions found in mass spectra of methyl esters of compounds I, II, and III after hydrogenation (compare Fig. 3)

Formation of Fragment Ion	I (M = 302)	II (M = 316)	III (M = 330)
M − CH_3O	271	285	299
M − CH_3OCO	243	257	271
M − CH_3OCOCH_2	229	243	257
M − CH_3O − [CH_3OCO + H]	211	225	239
M − CH_3O − [CH_3OCOCH_2 + H]	197	211	225
M − CH_3O − CH_3OH − [CH_3OCO + H]	179	193	207
$CH_3OCO-(CH_2)_5-COOCH_3^+$	188	188	188
188 − CH_3OH	156	156	156
188 − [CH_3OCO + H]	128	128	128
188 − CH_3OH − CH_3OH	124	124	124
$CH_3OCO-(CH_2)_6-COOCH_3^+$		202	
202 − CH_3OH		170	
202 − [CH_3OCO + H]		142	
202 − CH_3OH − CH_3OH		138	
$CH_3OCO-(CH_2)_7-COOCH_3^+$			216
216 − CH_3OH			184
216 − [CH_3OCO + H]			156
216 − CH_3OH − CH_3OH			152

columns, as shown in Table 1. For the final identification we had 1.0 mg of compound *I*, 1.1 mg of compound *II*, and 4.5 mg of compound *III*. Thin-layer chromatography and gas–liquid chromatography indicated that these compounds contained less than 3% of impurities that could have been detected with these procedures.

Structure determination

The mass spectra of the methyl esters of compounds, *I, II,* and *III* are shown in **Fig. 2**, and an interpretation of the observed ions is given in **Table 2.** No molecular ions were found, but it was possible to explain all the observed ions by well-known fragmentation mechanisms, from the assumed molecular masses of 300, 314, and 328 daltons. Aliphatic methyl esters do not always produce visible molecular ions on electron impact mass spectrometry. Pyrrolidides, prepared from fatty acids (4), have given visible molecular ions. However, several attempts to prepare such derivatives from the present compounds were unsuccessful, although monopyrrolidides were formed in our hands from a number of aliphatic dicarboxylic acids.

The main type of fragmentation seen in these spectra was the loss of methoxy groups, carboxymethyl groups, and carboxymethyl groups plus one or a few adjacent methylene groups. One series of ions was formed by the loss of one of these groups, another series of ions by the loss of two of these groups, and a third series of ions by the loss of three of these groups (in most of the possible combinations, see Table 2). Therefore, compounds *I–III* must contain three carboxy-methyl groups. This interpretation gained support from the mass spectrum of the CD_3-ester of compound *III*. In this spectrum, we found an increment of six mass units for all ions formed by the loss of one methoxy or carboxymethyl group, and an increment of three mass units for all ions formed by the loss of two such groups. No mass shift was observed for any of the ions due to loss of three such groups.

No changes in the mass spectra were noted after treatment of the methyl esters with silylation reagent. This excluded the presence of hydroxyl groups. The presence of ketonic functions was also ruled out since treatment with hydroxylamine-HCl followed by silylation did not result in any changes in the mass spectra. Based on this, we concluded that the parent compounds had the formulas $C_9H_{15}(COOH)_3$ (*I*), $C_{10}H_{17}(COOH)_3$ (*II*), and $C_{11}H_{19}(COOH)_3$ (*III*). Fully saturated aliphatic compounds would contain two more hydrogens. This indicated the presence of a double bond or a ring structure.

The mass spectra were changed after catalytic hydrogenation of the methyl esters (see **Fig. 3** and **Table 3**). As before, no molecular ions could be found. However, the ions found in the mass interval *m/e* 200–300 were easily derived from assumed molecular masses of 302, 316, and 330 daltons, by fragmentations at one, two, or three carboxymethyl groups. The result thus showed the existence of a double bond. In addition, the fragmentation pattern now revealed the branching point of the carbon

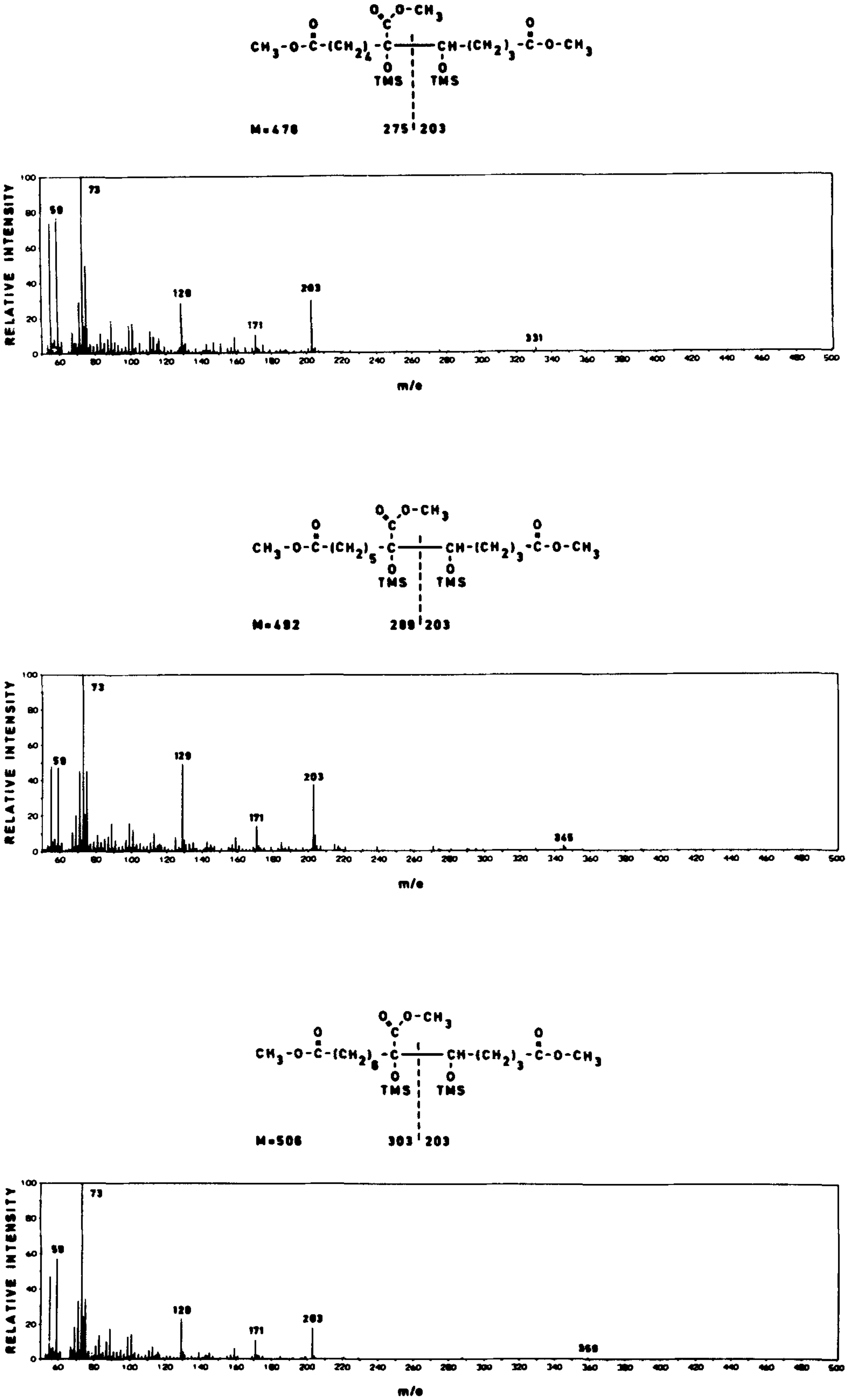


Fig. 4. Mass spectra of the methyl esters of compounds *I* (top spectrum), *II*, and *III* after oxidation with osmium tetroxide and trimethylsilylation. For interpretation, see Table 4.

TABLE 4. Ions found in mass spectra of trimethylsilylated, hydroxylated derivatives of methyl esters of compounds I, II, and III (compare Fig. 4)

Formation of Fragment Ion	I (M = 478)	II (M = 492)	III (M = 506)
M − [$(CH_3)_3Si$ + CH_3 + CH_3OCO]	331	345	359
See formula in Fig. 4	203	203	203
203 − CH_3OH (m* = 144.0)	171	171	171
203 − [CH_3OCOCH_2 + H]	129	129	129
$(CH_3)_3$ − Si	73	73	73
CH_3OCO	59	59	59

skeleton. The formulas given in **Fig. 4** are the only ones compatible with such ions as *m/e* 188 (compound *I*), *m/e* 188 and 202 (compound *II*) and *m/e* 188 and 216 (compound *III*). Another distribution of the methylene groups between the branches would cause the formation of other ions that were not found.

In order to determine the double bond position, methyl esters of compounds *I* to *III* were treated with osmium tetroxide, and the diols were trimethylsilylated. The resulting mass spectra are shown in Fig. 4 and an interpretation is given in **Table 4.** The large ion at *m/e* 203 showed that the double bond was separated by three methylene groups from one of the carboxyl groups. A fragmentation between two vicinal trimethylsilyloxy groups will usually give charge retention on both sides of the bond, but no ions were found at *m/e* 275, 289, and 303 (compare Fig. 4). Probably, these ions were unstable due to the closely attached carboxymethyl groups. The ions at *m/e* 331, 345, and 359, formally M − 147, were formed by the loss of one carboxymethyl group, one trimethylsilyl group, and one methyl group from the remaining trimethylsilyl group. This interpretation was based on the mass spectrum of the derivative with deuterium-labeled trimethylsilyl groups. In this spectrum, the ion at *m/e* 359 (compound *III*) was shifted to *m/e* 365. The ions at *m/e* 203, 171, 129, and 73 showed increments of nine daltons, as expected.

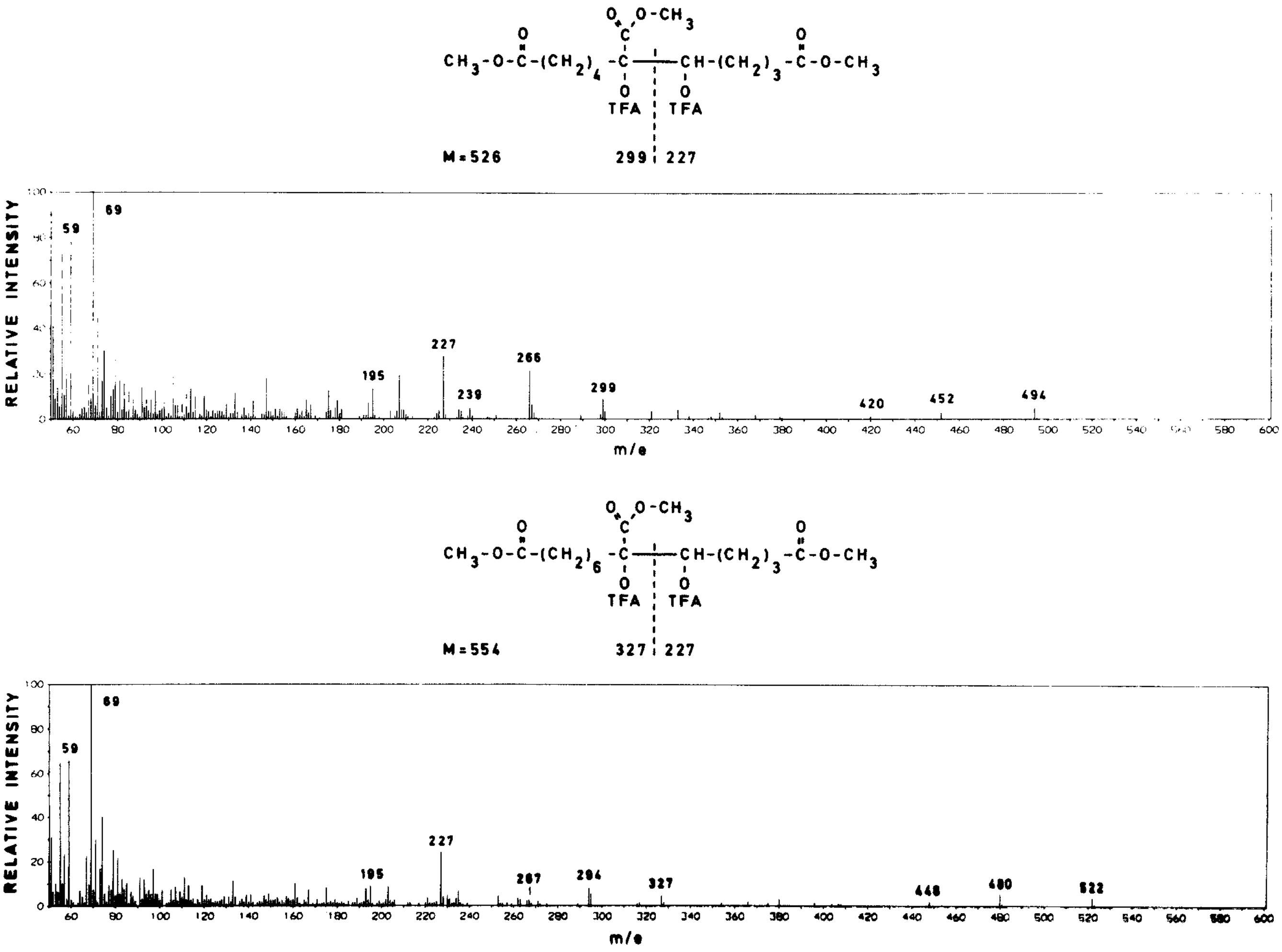


Fig. 5. Mass spectra of the methyl esters of compounds *I* (top spectrum) and *III* after oxidation with osmium tetroxide and trifluoroacetylation. For interpretation, see Table 5.

TABLE 5. Ions found in mass spectra of trifluoroacetylated, hydroxylated derivatives of methyl esters of compounds I and III (compare Fig. 5)

Formation of Fragment Ion	I M = 526	III M = 554
M − CH_3OH	494	522
M − [CH_3OCOCH_2 + H]	452	480
M − CH_3OH − [CH_3OCOCH_2 + H]	420	448
See formula in Fig. 5	299	327
299 or 327 − [CH_3OH + H]	266	294
299 or 327 − [CH_3OCO + H]	239	267
See formula in Fig. 5	227	227
227 − CH_3OH	195	195
CF_3 −	69	69
CH_3OCO	59	59

The compounds were also treated with trifluoroacetylation reagent after osmium tetroxide oxidation. Mass spectra from these derivatives of compounds *I* and *III* are shown in **Fig. 5**, and an interpretation is given in **Table 5.** Cleavage between the vicinal trifluoroacetoxy groups now resulted in charge retention on both of the two fragments, which were visible at *m/e* 227 and 299 for compound *I* and at *m/e* 227 and 327 for compound *III*. The corresponding mass spectrum from compound *II* was of poor quality due to lack of substance, but ions at *m/e* 227 and 313 were clearly visible. No molecular ions were seen, but the sum of the masses for the two parts formed by cleavage of the acylated diols gave the expected masses of 526, 540, and 554 daltons.

Although the mass spectra had shown the double bond to be in Δ5 position to one of the (two possible) carboxyls, its location in the carbon chain had not been established. The infrared spectrum of the methyl ester of compound *III*, before and after catalytic hydrogenation, is shown in **Fig. 6.** The corresponding spectra for compounds *I* and *II* were similar. Before hydrogenation, the carbonyl stretching vibration is split into two bands, one more intense at 1745–1750 cm^{-1} (nonconjugated C = 0) and another at 1725 cm^{-1} (conjugated C = 0). This spectrum also contained a small but distinct absorbance at 1650 cm^{-1} due to a conjugated double bond. As expected the bands at 1725 cm^{-1} and 1650 cm^{-1} disappeared after hydrogenation. It may be concluded that the double bond must be located in the shorter methylene chain. Otherwise, it would not be in conjugation with one carbonyl, and at the same time in Δ5-position to one of the remaining two carboxyl groups. The complete structure is therefore that shown in Fig. 2 above the mass spectra.

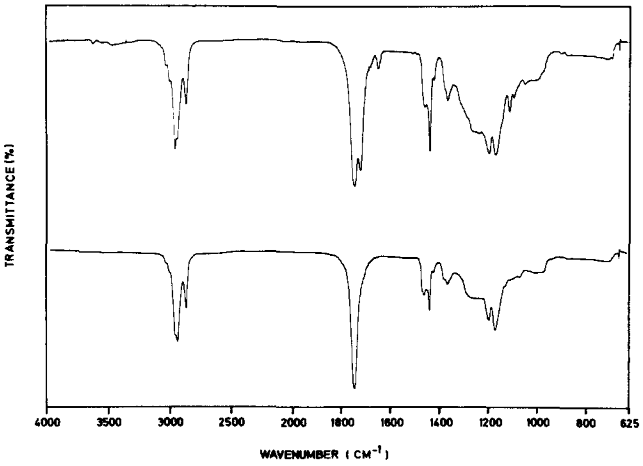
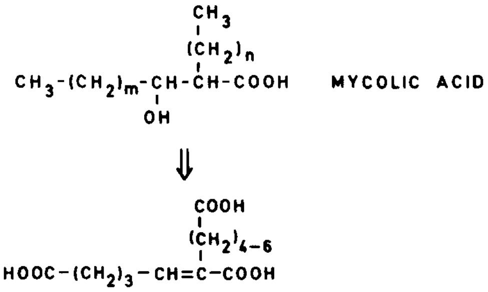


Fig. 6. Infrared spectrum of the methyl ester of compound *III*, before (top spectrum) and after (bottom spectrum) catalytic hydrogenation.



$$CH_3\text{-}(CH_2)_m\text{-}\underset{OH}{CH}\text{-}\overset{CH_3\text{-}(CH_2)_n}{CH}\text{-}COOH \quad \Downarrow \quad HOOC\text{-}(CH_2)_3\text{-}CH{=}\overset{COOH\text{-}(CH_2)_{4-6}}{C}\text{-}COOH$$

Fig. 7. Proposed metabolic relation between a mycolic acid and an aliphatic tricarboxylic acid.

Amount excreted and dietary origin

The average 24-hr excretion for one rat was 0.3–0.5 μmoles for each of the three compounds. The acids were constantly found as long as the rats were fed Astra-Ewos standard pellets, but fell to undetectable levels when this was replaced with sucrose or white bread and water. The acids could not be detected in pellet extracts.

DISCUSSION

Tricarboxylic acids with aliphatic chains of the length observed in this study are unusual compounds, but have been found in a few cases. From the eggs of certain hard ticks (*Dermatocenter andersoni*), a C_{15} tricarboxylic acid was isolated which made up 15% of the total fatty acids (5). The acid was an *iso*-methyl branched fatty acid that had two vicinal carboxyl groups in the middle of the chain; the exact position was not reported. The published mass spectrum of the methyl ester of this acid is different in many ways from our spectra. Another type of tricarboxylic acid has been isolated from basidiomycetes such as

Polyporus and *Cantharellus floccosus* (6, 7). The major examples are 2-tetradecylcitric acid (norcaperatic acid) and 2-hexadecylcitric acid (agaricic acid). These acids are pharmacologically active and cause gastrointestinal disturbances due to an atropine-like anticholinergic effect. None of the previously known aliphatic tricarboxylic acids have any structural resemblance to the compounds reported here.

The acids were obviously of dietary origin. It is attractive to suggest that they were formed from fatty acids with a long branch on carbon atom 2, so called mycolic acids (see **Fig. 7**). Acids of this type have so far been described as constituents of *Mycobacteria*, *Nocardia*, and *Corynebacteria* (see ref. 8, 9 and references cited therein). This type of fatty acid has one hydroxyl at carbon atom 3. Sometimes the major aliphatic chain may contain double bonds, cyclopropane rings, or methyl branches. Though nothing is known about the mammalian metabolism of mycolic acids, one would expect ω-oxidation to occur at the methyl end of each aliphatic chain, followed by β-oxidation (Fig. 7). Dehydration would result in the type of acids we have found. The parent acids may also have a double bond in the 2 position although such mycolic acids have not been reported.

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