Journal of Chromatography, 479 (1989) 61–72
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 719

CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY AND IDENTIFICATION OF SUBSTITUTED CARBOXYLIC ACIDS IN LIPIDS EXTRACTED FROM A 4000-YEAR-OLD NUBIAN BURIAL

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(First received April 10th, 1989; revised manuscript received June 19th, 1989)

SUMMARY

Polar carboxylic acid fractions of lipids extracted from samples from a 4000-year-old Nubian mummy were investigated by capillary gas chromatography–mass spectrometry. They contain series of α,ω-dicarboxy, monohydroxy, dihydroxy and keto fatty acids, some of which were found for the first time outside the synthetic laboratory. Their chromatographic and mass spectrometric characteristic properties are reported.

INTRODUCTION

During an investigation of the extractable lipids from diverse parts of a 4000-year-old Nubian burial belonging to the Ancient Kerma[1] civilization, the substituted fatty acids in the polar acid fractions were examined by gas chromatography–mass spectrometry (GC–MS). In addition to a homologous series of C_7–C_{18} α,ω-dicarboxylic aliphatic acids and diverse C_{16}–C_{20} mid-chain vicinal dihydroxy fatty acids, we identified several homologous series of C_7–C_{18} monohydroxy fatty acids containing every positional isomer except the 4-hydroxy acids, and a series of all keto fatty acids excluding the α-, β-, $(\omega-2)$- and $(\omega-3)$-oxo positional isomers. Moreover, a homologous series of *n*-alkylsuccinic acids with alkyl substituents ranging from C_{11} to C_{16} were also found in the extract from inside of the skull. We report here the GC retention characteristics of these compounds on an OV-73 coated capillary column and their mass spectrometric fragmentation patterns.

0021-9673/89/$03.50

EXPERIMENTAL

Samples, extraction and preparation

The mummy, of a 40–50-year-old male, was found in a necropolis at Kerma in Upper Nubia (Sudan) excavated in 1983.

Samples of skin and concretions from the inside of the skull and of the thorax were extracted by homogenizing the tissue with chloroform–methanol (2:1) and the extracts were kept in a refrigerator until analysis. The extracted lipids were separated into various classes using chromatographic procedures that have already been described for geological[2,3] or biological[4] organic material. Briefly, the lipids were first separated into neutral and acidic fractions by chromatography on a silica gel column (200 x 12 mm I.D., 70–230 mesh, 10% potassium hydroxide). The acidic fraction was esterified with 14% boron trifluoride in methanol and the resulting methyl esters were separated by flash chromatography[5] on a second silica gel column (300 x 6 mm I.D., 70–230 mesh, nitrogen pressure 0.3–0.5 bar). After the elution of the monocarboxylic fatty acid methyl esters with 50 ml of hexane–methylene chloride (3:1), the polar carboxylic acid methyl esters were eluted with 50 ml of methylene chloride–ethyl acetate (9:1). Before the GC–MS analyses were performed, the polar acid fraction was trimethylsilylated at 70–90°C for 15 min with bis(trimethylsilyl)trifluoroacetamide containing 1% of trimethylchlorosilane (Fluka, Buchs, Switzerland).

GC–MS analyses

The equipment used was a Finnigan 4000 quadrupole mass spectrometer equipped with the SuperIncos data system and coupled to a Carlo Erba 5300 Mega Series gas chromatograph. Analyses were performed with laboratory made glass capillary columns (Duran, 25 m x 0.32 mm I.D.), coated with OV-73 (Alltech). After splitless injection at 60°C, the GC oven was heated to 130°C at 10°C/min and then to 280°C at 4°C/min. The detailed GC and MS conditions have been described elsewhere[2]. Equivalent chain lengths (ECL) of the unbranched α,ω-dicarboxylic acid dimethyl esters were first determined by coinjection of a sample with a standard mixture of *n*-alkanoic acid methyl esters and found to be equal to $(n + 3) + 0.33$ (± 0.04) where n is the number of carbon atoms in the free diacid. The ECL values of the other polar acids were calculated on the basis of their retention times relative to those of the α,ω-dicarboxylic acids which were present in all samples from the mummy.

RESULTS AND DISCUSSION

Fig. 1 shows the reconstructed total ion chromatogram (TIC) of the methylated and trimethylsilylated polar acid fraction separated from white concretions found inside the mummy's thorax. The early eluting part of the chromatogram is dominated by regularly spaced peaks arising from the saturated straight-chain α,ω-dicarboxylic acid dimethyl esters (C_7–C_{18}) identified on the basis of their mass spectra. The compounds of this series exhibit in their mass spectra[6] and intense m/z 98 peak which was used for their detection by mass fragmentography[7].

Between two successive homologues of the α,ω-dicarboxylic acids, the TIC shows a very complex pattern, especially in the late-eluting part, as exemplified by the

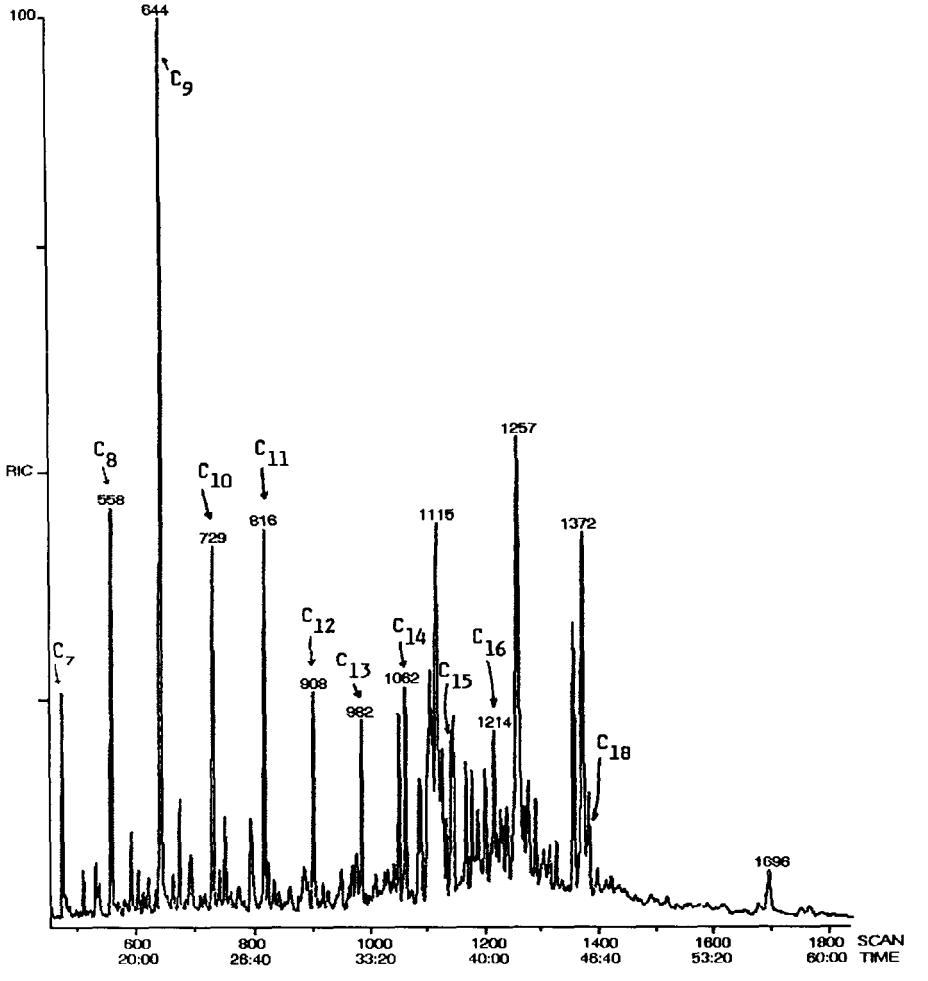


Fig. 1. Reconstructed total ion chromatogram of the esterified and trimethylsilylated polar acid fraction from the thorax extract (peaks marked C_n represent α,ω-dicarboxylic acids with n = number of carbon atoms in the free acid).

partial TIC in Fig. 2. A close examination of the mass spectra showed that most of the peaks appearing as single chromatographic peaks on this trace are in fact due to several coeluting components. Almost all components of this chromatogram were identified by comparison of their mass spectra and retention times with those of authentic analogues, with published spectra of lower homologues or by the interpretation of their mass spectra, as shown in Tables I and II.

Oxo fatty acids

The major oxo fatty acids included a homologous series of 4-oxo fatty acids in the C_{11}–C_{18} range with the C_{16} homologue being the most abundant. They are characterized by a major McLafferty rearrangement ion at m/z 130 and a secondary

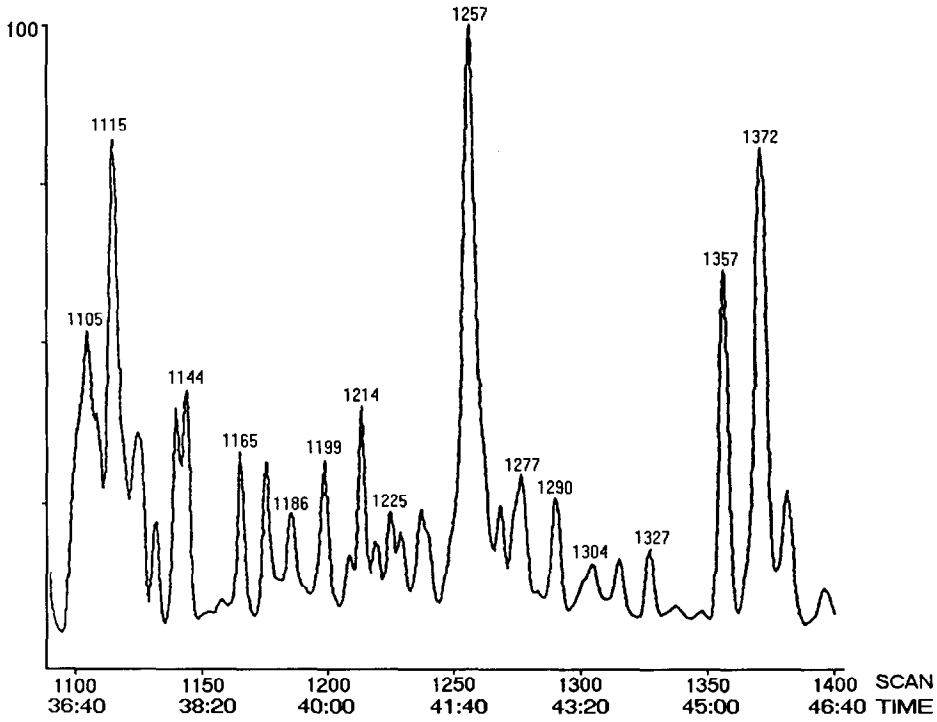


Fig. 2. Detail of part of the reconstructed total ion chromatogram of Fig. 1.

TABLE I

KETO ACIDS FOUND IN LIPIDS EXTRACTED FROM THE MUMMY

See Experimental for GC–MS conditions.

Compound	*Chain length (n)*	*ECL*	*Fragment*[a]	*Other peaks*[b]
4-Oxo	8–20	$(n+1)+0.61$	98, 130	115, (M − 31),(M − 87)
5-Oxo	9–20	$(n+1)+0.65$	144	112,129,(M − 31),(M − 101)
6-Oxo	12–18	$(n+1)+0.79$	158	126,143,(M − 31),(M − 115)
7-Oxo	12–18	$(n+1)+0.82$	172	(M − 114),(M − 31)
8-Oxo	13–18	$(n+1)+0.84$	186	(M − 128),(M − 31)
9-Oxo	16–18	$(n+1)+0.86$	200	(M − 142), (M − 31)
10-Oxo	16–18	$(n+1)+0.87$	214	(M − 156),(M − 31)
11-Oxo	16–18	$(n+1)+0.88$	228	(M − 170)
12-Oxo	16–18	$(n+1)+0.89$	242	(M − 184)
13-Oxo	18	$(n+1)+0.90$	256	(M − 198)
$(\omega-1)$-Oxo	8	$(n+2)+0.07$	58	71,(M − 89),(M − 57),(M − 31)
$(\omega-1)$-Oxo	to 18	$(n+2)+0.16$	58	71,(M − 89),(M − 57),(M − 31)

[a] m/z of ion(s) used for fragmentographic detection.
[b] m/z of other diagnostic ions, not necessarily major ones.

TABLE II

ECL VALUES OF MONOHYDROXY FATTY ACIDS (AS METHYL ESTER TMS ETHERS) FOUND IN THE EXTRACTS FROM THE MUMMY

See Experimental for GC–MS conditions. See text for ions a and b.

Chain length	*m/z of ion b and OH position*															
	103 ω	*117* $(\omega-1)$	*131* $(\omega-2)$	*145* $(\omega-3)$	*159* $(\omega-4)$	*173* $(\omega-5)$	*187* $(\omega-6)$	*201* $(\omega-7)$	*215* $(\omega-8)$	*229* $(\omega-9)$	*243* $(\omega-10)$	*257* $(\omega-11)$	*271* $(\omega-12)$	*285* $(\omega-13)$	*299* $(\omega-14)$	*301* $(\omega-15)$	*315* $(\omega-16)$
7	10.76	10.09															
8	11.72	11.03	10.91			10.39	10.39										
9	12.70	11.97	11.86	11.62			11.31	11.31									
10	13.64	12.95	12.83	12.58	12.45			12.21	12.21								
11	14.54	13.93	13.80	13.56	13.46	13.34			13.18	13.18							
12	15.52	14.91	14.77	14.51	14.41	14.30	14.24			14.12	14.12						
13	16.51	15.89	15.75	15.48	15.39	15.25	15.18	15.16			15.08	15.08					
14	17.50	16.87	16.73	16.46	16.29	16.22	16.15	16.12	16.12			16.05	16.05				
15	18.47	17.84	17.70	17.42	17.27	17.17	17.12	17.09	17.09	17.09			17.02	17.02			
16	19.51	18.80	18.66	18.37	18.21	18.14	18.05	18.02	17.98	18.01	18.02			17.97	17.97		
17		19.77	19.63	19.36		19.09	19.01			18.95					18.94	18.94	
18	21.46	20.73	20.60	20.33	20.15	20.03	19.97	19.91	19.89	19.88	19.87	19.89	19.90	19.93		19.89	19.89
m/z of ion a[a]	–	371	357	343	329	315	301	287	273	259	245	231	217	203	189	175	Absent[b]
OH position[a]	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2

[a] Diagonal entries towards the top and left side of the table.

[b] α-Hydroxy acids are detected by fragmentograms of *m/z* 159 and (M − 59).

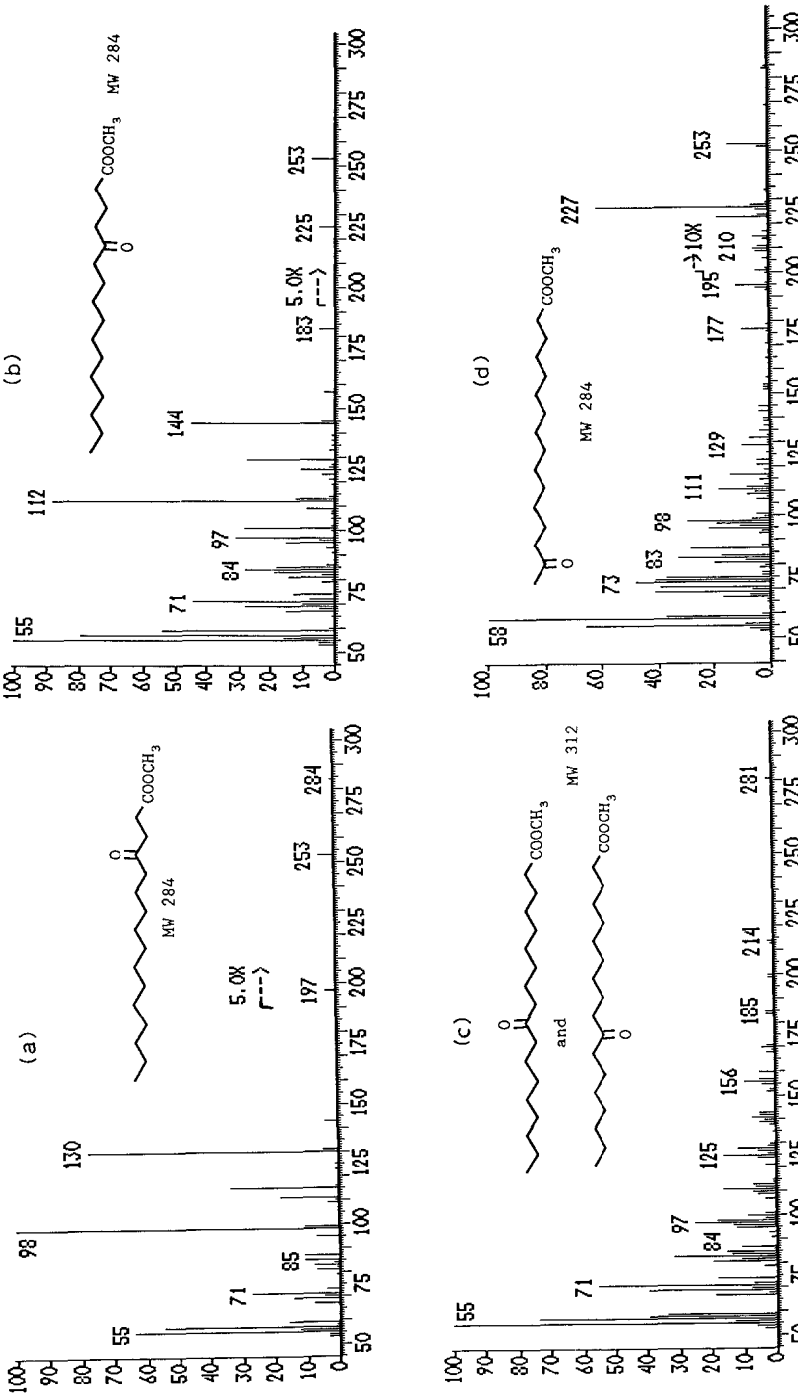


Fig. 3. Mass spectra of methyl esters for (a) 4-oxohexadecanoic acid, (b) 5-oxohexadecanoic acid, (c) mixture of 9- and 10-oxooctadecanoic acids and (d) 15-oxohexadecanoic acid found in the mummy extracts.

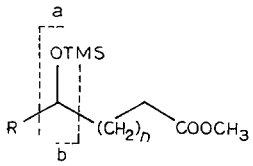
fragment at m/z 98 (130 − CH_3OH), and also by the α-cleavage ions at m/z 115 and m/z 197 (M − 87), as shown in the mass spectrum of the 4-oxohexadecanoic acid illustrated in Fig. 3a. Thus, 4-oxo fatty acids may also be detected by the m/z 98 mass fragmentogram as they are chromatographically well separated from the dicarboxylic acids. The 4-oxo acids are accompanied by smaller amounts of the corresponding 5-oxo isomers which are characterized by a similar fragmentation pattern [McLafferty ion at m/z 144, m/z 112 (144 − CH_3OH) and α-cleavage ions at m/z 129 and m/z (M − 101)] (Fig. 3b). They elute just after the 4-oxo isomers as only partially resolved peaks. The other prominent oxo-acids identified were coeluting mixtures of 9- and 10-oxo isomers of hexadecanoic and octadecanoic acids. The mass spectra of these isomers are also characterized by the same fragments as those produced from the 4- and 5-oxo isomers, but these fragments, although of diagnostic value, are not the major ones (Fig. 3c).

Another relatively important homologous series of keto acids is the one with the oxo function on C-(ω − 1). These acids have much higher ECL values than those of the mid-chain keto acids of the same chain length and their mass spectra are characterized by an intense m/z 58 ion from McLafferty rearrangement (Fig. 3d). Finally, minor or trace amounts of keto fatty acid positional isomers with the keto function located between C-6 and C-(ω − 4) were observed on the mass fragmentograms of the corresponding McLafferty rearrangement ions, as shown in Table I.

Oxo fatty acids with the keto function at a given position show a constant increase in their ECL value with increasing carbon number, except when the function is on the (ω − 1) carbon atom. Thus, a 4-oxo fatty acid methyl ester will elute (on the GC time scale) at a position corresponding to 61% of the distance between the elution positions of the *n*-fatty acid methyl esters with one and two more carbon atoms, respectively (Table I). If we call this a "constant increment", then the (ω − 1)-oxo fatty acids show a slightly "positive increment" of their ECL with increasing carbon number.

Monohydroxy fatty acids

The mass spectra of the two large peaks (*i.e.*, 1115 and 1257) in the chromatogram in Fig. 2 showed that they are produced by unresolved isomers of monohydroxypalmitic and monohydroxystearic acids, respectively. The mass spectra of monohydroxy fatty acid TMS ether methyl ester derivatives are characterized by intense fragments from α-cleavage to the OTMS group, specific to the position of the original hydroxy group[8,9], except for α- and ω-hydroxy acids which do not show ion *a*:



The other characteristic ions which are common to all spectra are m/z 73 (TMS group), the (*a* − 29) ion produced by the loss of CHO from ion *a* after migration of the TMS group to the carboxylic carbonyl group and the (*a* − 32) ion

($[a-CH_3OH]^+$). Table II gives the ECL values of all the monohydroxy acids found in the mummy samples and the m/z values of the ions a and b used for their detection. The ω-hydroxy acids which do not exhibit ion a were detected by the $(M-47)$ ions[8]. Every positional isomer of the monohydroxy acids in the range C_7–C_{18}, except the 4- and 5-hydroxy isomers, was present. The α- and β-hydroxy isomers having the same carbon number coelute in the entire carbon number range but the contribution of the α-hydroxy acids may be calculated through the abundance of the $(M-59)^+$ ion, present in their mass spectra[8]. The other positional isomers are all resolved for the acids with less than 13 carbon atoms. Acids with longer chains show either coelution or only partial resolution of the isomers bearing the hydroxyl group on carbon atoms α- to $(\omega-6)$ positions. ECLs of homologous acids in the series with the hydroxy substituent on a given carbon atom (from the carboxylic carbon side) clearly exhibit a "negative increment" with increasing chain length (Table II, diagonal entries). Two higher α-hydroxy acid homologues, C_{22} and C_{23} (not shown in Table II), were also found in significant amounts in all the extracts.

Dihydroxy fatty acids

Both the large peaks at 1357 and 1372 in the TIC in Fig. 2 show almost identical mass spectra (Fig. 4a); they were identified as the *threo* and *erythro* isomers

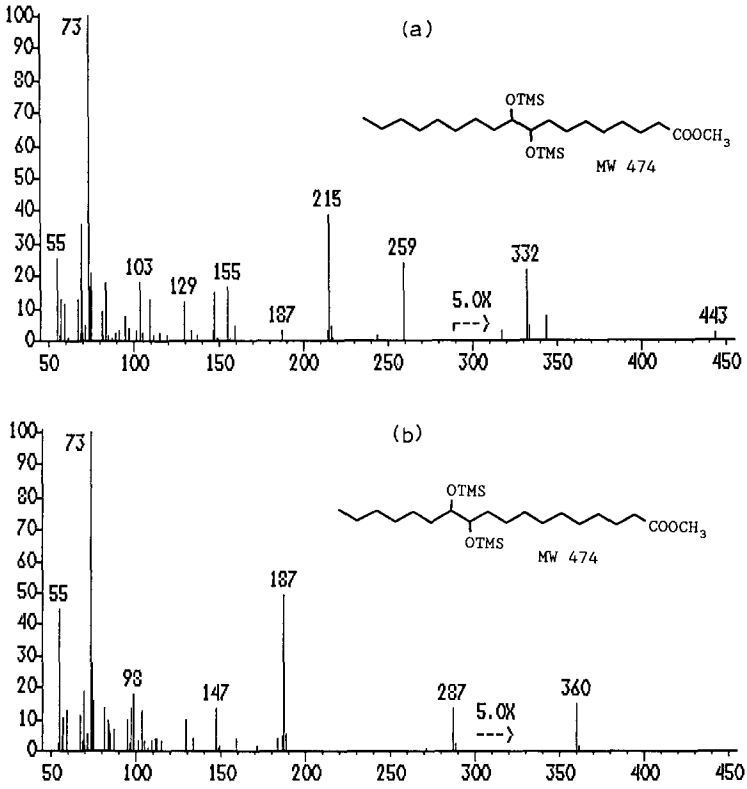
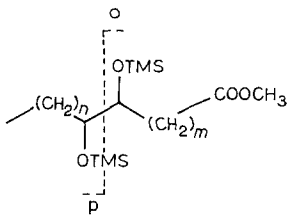


Fig. 4. Mass spectra of methyl ester TMS ethers for (a) 9,10-dihydroxyoctadecanoic acid and (b) 11,12-dihydroxyoctadecanoic acid found in the mummy extracts.

of methyl 9,10-(ditrimethylsilyloxy)octadecanoate[10], respectively. The mass spectra of these compounds are characterized by the two intense *o* and *p* ions, corresponding to the cleavage of the bond linking the carbon atoms which bear the OTMS groups, with retention of the charge on either side[8,10], as shown below.



The other characteristic ions in the mass spectra of mid-chain vicinal dihydroxy acids are *m/z* 73 (base peak, TMS), *m/z* 147 and *m/z* 159, which are common to all spectra and the (M − 15), (M − 31) and [M − $CH_3(CH_2)_nCHO$] ions, which are of diagnostic value despite their low intensities ($\leqslant 3\%$ of the base peak). Other vicinal dihydroxy fatty acids, identified on the basis of their mass spectra, are listed in Table III, and the mass spectrum of 11,12-dihydroxyoctadecanoic acid is shown in Fig. 4b.

The abundance ratio of the *threo* and *erythro* isomers, as measured from the intensities of ions o, are very significantly dependent on the origin of the sample. In the thorax extract, for example, the *erythro* isomer of the 9,10-dihydroxy C_{18} acid is approximately twice as abundant as the *threo* isomer, whereas in the extract from the skull it is the *threo* isomer that is more prominent; in the skin the two enantiomers show similar abundances.

TABLE III

ECL VALUES OF DIHYDROXY FATTY ACIDS (AS METHYL ESTER TMS ETHERS) FOUND IN THE EXTRACTS FROM THE MUMMY

See Experimental for GC–MS conditions.

Chain length	*Position of OH groups*	*Diagnostic ions*		*ECL*
		o	*p*	
16	6,7 *threo*	217	229	19.24
16	6,7 *erythro*	217	229	19.40
16	7,8 *threo*	231	215	19.24
16	7,8 *erythro*	231	215	19.40
16	9,10 *threo*	259	187	19.29
16	9,10 *erythro*	259	187	19.44
17	8,9 *threo*	245	215	20.13
17	8,9 *erythro*	245	215	20.29
17	10,11 *threo*	273	187	20.23
17	10,11 *erythro*	273	187	20.41
18	9,10 *threo*	259	215	21.05
18	9,10 *erythro*	259	215	21.22
18	11,12 *threo*	287	187	21.17
18	11,12 *erythro*	287	187	21.33

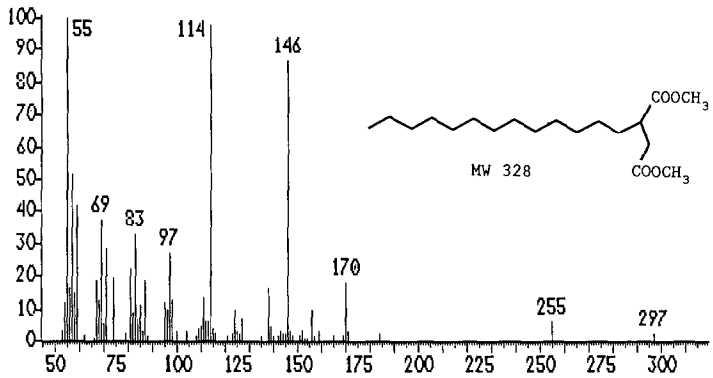
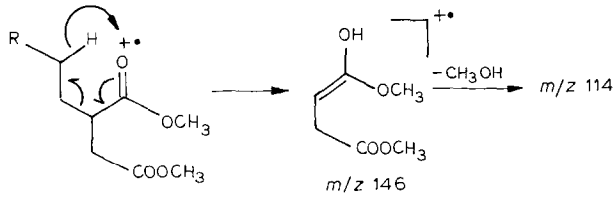


Fig. 5. Mass spectrum of the methyl ester of *n*-tridecylsuccinic acid found in the extract from the skull.

Alkylsuccinic acids

The mass spectrum of a relatively intense peak eluting at an ECL value of 18.84 in the TIC of the skull extract is shown in Fig. 5. The major ions at *m/z* 146 and 114 can be interpreted as originating from an alkyl-substituted succinic acid dimethyl ester by a McLafferty rearrangement and a subsequent loss of CH_3OH:



Indeed, a comparison of the spectrum in Fig. 5 with that of methyl *n*-dodecylsuccinate[11] confirms the unknown compound as being a C_{13}-alkyl-substituted succinic acid. A search for the occurrence of other homologues in the extract by means of *m/z* 146 and 114 fragmentograms indicated the presence of C_{11}- (ECL = 16.88), C_{12}- (17.86), C_{14}- (19.82), C_{15}- (20.79) and C_{16}- (21.79) alkylated analogues. The evolution of the ECL values suggests that the alkyl substituent is linear. These compounds could not be detected in the skin or thorax samples.

DISCUSSION

Most of the substituted fatty acids founds in the extracts from the mummy are normally not found in human or animal tissues. They are obviously produced by *post mortem* transformations of the original fatty acids. As will be reported elsewhere[12], other lipid compounds such as the sterols, the bile acids and, more particularly, the unsubstituted monocarboxylic acids also showed more or less intense modifications. The last group included only saturated fatty acids (indicating a complete disappearance of the unsaturated fatty acids normally found in human tissues), comprising *iso-*

and *anteiso*-methyl-branched homologues in the C_{13}–C_{19} range. This suggests a bacterial reworking of the original carboxylic acids. The relative abundances of the diverse substituted acids reported here suggest that most of them are produced by oxidation of the double bond of an unsaturated fatty acid originally present in the body. Thus, in the α,ω-dicarboxylic acid series, the most abundant compound is the C_9 homologue; in the mid-chain monohydroxy or keto acid series, those with 18 and 16 carbon atoms and the oxo function on C-9 and C-10 are the most abundant. In the dihydroxy fatty acid series, 9,10-dihydroxy acids, presumably produced by dihydroxylation of the double bonds of oleic and palmitoleic acids, are major compounds.

Among the acids reported here, the complete series of isomeric monohydroxy and keto acids have never been reported in natural samples, although recently they have been detected in aerosols in the C_6–C_{15} range, with maxima at C_9 or C_{11}[9,13]. The 10-oxo- and 10-hydroxy C_{18} and C_{16} acids have been reported in the adipocere from human bodies having been immersed in the sea for 3–6 months[14,15], but positional isomers and other homologues were not mentioned.

Mid-chain vicinal dihydroxy acids are well known compounds which are routinely prepared from unsaturated fatty acids in order to determine the position and the geometry of the double bonds[10,16] in the original compounds. However, to our knowledge, they have never been reported as occurring in samples from outside the laboratory. As we already pointed out, in the mummy samples they are obviously produced through dihydroxylation of the double bond in a suitable precursor. It is interesting that the enantiomeric composition of these acids is dependent on their origin. Two possibilities may explain this: (i) the original unsaturated precursor acids had the same double bond geometry but different mechanisms of dihydroxylation were operative, or (ii) the mechanism of formation was the same but the ratio of the *cis*- to *trans*-unsaturated acids in diverse parts of the body was different.

The mass spectrometric fragmentation patterns of the *n*-alkylsuccinic acids have been interpreted by using spectra obtained from methyl and ethyl esters of synthetic *n*-dodecylsuccinic acid[11]. The only report on the occurrence of this type of compound in a natural sample concerns Green River oil shale, a 60 million-year-old sediment, in which a homologous series with an alkyl substituent ranging from C_{13} to C_{29} (C_{18} and C_{28} members absent) was detected[17]. The fact that in the mummy they occur only in the skull extract and that only the C_{13} alkyl-substituted homologue is of significant abundance, must have either a biochemical or a diagenetic significance, but at present we have no explanation.

ACKNOWLEDGEMENTS

This work was supported in part by the Fonds National Suisse de la Recherche Scientifique (Grant No. 2000-5.222). We thank Mrs. O. Vaucher for valuable technical assistance.

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