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A New Series of Long-Chain Dicarboxylic Acids with Vicinal Dimethyl Branching Found as Major Components of the Lipids of *Butyrivibrio* spp.

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1. Some members of the genus *Butyrivibrio*, including a general fatty acid auxotroph (strain S2), contain as a major part of their complex lipids a high-molecular-weight component that is probably formed by the union of two fatty acid chains [Hazlewood & Dawson (1979) *J. Gen. Microbiol.* **112**, 15–27]. 2. Proton and ^{13}C n.m.r. and i.r. and mass spectroscopy were used to examine a homologous series of these moieties and, in addition, the hydrocarbon derivative of one homologue and several synthetic compounds. 3. The results indicate that the high-molecular-weight components are a series of long-chain dicarboxylic acids containing vicinal dimethyl branching, located near the centre of the chain.

Members of the genus *Butyrivibrio* are among the most numerous and biochemically versatile groups of bacteria occurring in the rumen. Butyrivibrios participate in the digestion of cellulose, the hydrolysis of plant lipids, the hydrogenation of polyunsaturated fatty acids, the production of butyrate from carbohydrate and the interconversion of acetate and butyrate. Recently we have isolated from the rumen a *Butyrivibrio* sp. (strain S2) that has an absolute requirement for long-chain fatty acid (Hazlewood & Dawson, 1979).

An examination of the complex lipids of this organism showed that an appreciable percentage of the hydrocarbon chains consisted of a high-molecular-weight condensation product, probably formed from two molecules of fatty acid (Hazlewood & Dawson, 1979). Subsequently we detected similar substances in other members of the genus, including those that do not require fatty acid as an essential nutrient. In addition, evidence has recently been obtained that similar substances occur in the total lipids of mixed rumen micro-organisms and ruminant faeces.

In the present paper we provide evidence that the substances isolated are long-chain dicarboxylic acids with two adjacent methyl groups located in the central region of the chain. Their formation involves a reductive condensation reaction between two molecules of fatty acid, and a series of these dicarboxylic acids can be formed depending on the fatty acid(s) used to promote growth of *Butyrivibrio* S2. The determination of the basic structure proved to be difficult, since on mass-spectral analysis of the dimethyl esters of the dicarboxylic acids methanol rather than a methoxy group was lost from the molecular ion. Since, as indicated below, the loss of methanol is characteristic of methyl esters of unsaturated or cyclopropane fatty acids, whereas methoxy groups are lost from dimethyl esters of dicarboxylic acids (Ryhage & Stenhagen, 1959, 1960*a*,*b*), this observation proved to be very misleading. We propose therefore that the trivial name for this series of compounds (*vic*-dimethylalkanedioic acids) of the general formula:

$$\begin{array}{l} CH_3\text{–}CH\text{–}C_nH_{2n\ (or\ 2n-2)}\text{–}CO_2H \\ \quad\quad\quad | \\ CH_3\text{–}CH\text{–}C_mH_{2m\ (or\ 2m-2)}\text{–}CO_2H \end{array}$$

should be the diabolic acids from the Greek διαβολλω 'diabollo' (to mislead). We suggest also that the shorthand abbreviation of such compounds should be written in the form ($C_{m:x}$–$C_{n:y}$) diabolic acid, where x and y are the number of double bonds in each chain and m and n the number of carbon atoms, e.g. $C_{18:1}$–$C_{16:0}$ diabolic acid. When the molecule is symmetrical this can be abbreviated to $(C_{m:x})_2$ diabolic acid, e.g. $(C_{16:0})_2$ diabolic acid or $(C_{18:1})_2$ diabolic acid.

Materials and Methods

Isolation of dicarboxylic acid dimethyl ester

The auxotroph *Butyrivibrio* S2 was grown in a fatty acid-free basal medium under conditions that have been described previously (Hazlewood & Dawson, 1979). The fatty acid supplement (e.g.

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palmitic acid, stearic acid, octadec-*trans*-11-enoic acid, sometimes labelled with ^{14}C) was dispersed in sodium taurocholate solution and added at a final concentration of 30μg/ml. The cells were harvested after 18h incubation at 39°C (45°C for stearic acid-containing medium) by centrifugation (20000*g* for 20min at 5°C) and were extracted with lipid solvents as described by Clarke *et al.* (1976). The total lipids were methanolysed (2½h at 90°C) as described by Gray (1976) with 5% (w/v) HCl in anhydrous methanol. Hydrophobic substances were extracted from the hydrolysate in light petroleum (b.p. 40–60°C) after neutralization with anhydrous Na_2CO_3. The lipid products in the extract were applied as bands to preparative t.l.c. plates (Kieselgel F_{254}; Merck, Darmstadt, Germany) and separated by using light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (100:10:1, by vol.) as developing solvent. The plates were sprayed on one edge with dichlorofluorescein, or in experiments with radioactive precursor were radioautographed to locate dicarboxylic acid dimethyl esters (R_F 0.30), dimethylacetals (R_F 0.53) and methyl esters of fatty acids (R_F 0.63). The band corresponding to dicarboxylic acid dimethyl esters was scraped from the plate into a column fitted with a sintered disc and the material was eluted from the silica gel with diethyl ether and chloroform. Dicarboxylic acid dimethyl esters were recovered by evaporation, and were recrystallized from light petroleum (b.p. 40–60°C) at −20°C or from methanol. The dicarboxylic acid dimethyl ester derived from a palmitic acid growth supplement and recrystallized from methanol consisted of feathery needles melting at 42–43°C.

I.r. spectroscopy

The compound was smeared over an NaCl disc and examined in the Perkin–Elmer Infracord instrument. A more detailed spectrum was obtained from a Perkin–Elmer model 157G instrument with a 10% (w/v) solution of dicarboxylic acid dimethyl ester in carbon tetrachloride. Other spectra were recorded with the sample either as a film or as a KBr disc.

Mass spectrometry

Mass spectra were recorded on an Associated Electrical Industries MS902 instrument. Samples were admitted to the source region by using the direct-insert probe at approx. 200°C with an electron-beam energy of 70eV.

N.m.r. spectroscopy

Proton n.m.r. spectra were recorded at 100MHz on solutions (1–2%, w/v) in either [^2H]chloroform or carbon tetrachloride, with tetramethylsilane or chloroform being used for the lock signal. Fourier transform ^{13}C n.m.r. spectra were recorded at 200MHz on dilute solutions (1–2%, w/v) in [^2H]-chloroform, with a pulse delay of 10s and averaging between 1000 and 2000 scans.

Conversion of dicarboxylic acid dimethyl ester into parent hydrocarbon

Dicarboxylic acid dimethyl ester prepared from bacterial cells grown with palmitic acid was reduced by the use of $NaAlH_2(OCH_2CH_2OCH_3)_2$ (Red-al; Aldrich Chemical Co., Gillingham, Dorset, U.K.) (Snyder *et al.*, 1971). The diol product (9.8mg) was heated in a sealed tube with 1.5ml of 66% (w/v) HI for 4.5h at 128°C. After being cooled the reaction mixture was shaken with 15ml of diethyl ether and 5ml of water. The ether phase was separated and washed twice with 1M-NaOH (10ml) and thrice with water (10ml). The di-iodide formed was reduced by a method suggested by the work of Carey & Smith (1933). The ethereal solution of the di-iodide was dried overnight over $CaCl_2$, the ether removed and the residue dissolved in 6ml of acetic acid and heated under reflux. Zinc dust (0.5g) was added in small batches down the condenser over a period of 4.5h. At the end of the reduction, 20ml of water was added, and the hydrocarbon was extracted by vigorous shaking of the reaction mixture twice with 10ml portions of light petroleum (b.p. 40–60°C). The extract was evaporated to dryness and the residue re-extracted with 2ml of light petroleum. Evaporation produced a wax {7mg; $[\alpha]_D^{25}$ +2.74° (*c* 1.97 in chloroform); $[M]_D^{25}$ 12.33)}, which on examination by g.l.c. and t.l.c. [silica gel H; light petroleum (b.p. 40–60°C)/diethyl ether (10:1, v/v)] proved to be a hydrocarbon with little contamination by more polar compounds.

Synthesis of 12,13-*dimethyltetracosane*

2-Bromotridecane (10g) (Aldrich Chemical Co.) was treated with finely divided sodium metal (2g) in anhydrous di-n-butyl ether (200ml) at reflux temperature for 7h with vigorous stirring. At the end of the reaction period excess sodium was destroyed with ethanol, and the product was extracted with diethyl ether by removing the NaBr in solution with water. The viscous oil that remained on evaporation of the ether was subjected to fractional distillation under reduced pressure, yielding two major fractions. The first of these (3.46g; 53% of theoretical) had b.p. 93°C/0.4–0.5kPa (3–4mmHg) and n_D^{25} 1.4266, suggesting that it most probably consisted of a mixture of tridecenes [tridec-1-ene, b.p. 102°C/1.3kPa (10mmHg) and n_D^{20} 1.4328]. I.r. and n.m.r. spectroscopy confirmed this assumption. The second fraction (1.13g; 17% of theoretical) gave b.p. 216–220°C/0.4–0.5kPa (3–4mmHg) and n_D^{65} 1.4293. The literature values for the straight-chain homologue hexacosane are b.p. 205°C/0.1kPa (1mmHg), estimated 215–218°C/0.4–0.5kPa (3–4mmHg)] and n_D^{65} 1.43332. I.r. and n.m.r. spectroscopy confirmed that this

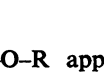
fraction was a saturated hydrocarbon. G.l.c. confirmed the chain length of the product. The retention index was determined as 2506±2 on 1.5m×6.3mm (5ft×¼in) columns of SE30 at 220°C; the straight-chain homologue would, by definition, have a retention index of 2600.

Minor unsaturated and coloured impurities were removed by brominating the product and eluting the non-brominated material from a column (1.8cm×32cm) of silicic acid (Mallinckrodt CC4) with light petroleum (b.p. 40–60°C).

Results and Discussion

I.r.-absorption spectrum

The spectrum of the unknown compound (dicarboxylic acid dimethyl ester) (from cells grown with palmitic acid) was very similar to published spectra of long-chain fatty acid methyl esters or long-chain dicarboxylic acid dimethyl esters with a carbonyl bond stretch at 1744cm^{-1} and stretching of the



carbon–oxygen bond C–O–R appearing between 1175 and 1250cm^{-1}. There was no evidence of unsaturation in the hydrocarbon chain or of oxygen combined in hydroxy or ether linkage. From the position of the carbonyl bond stretch it seemed unlikely that oxygen would occur as a constituent of a simple keto or aldehyde grouping.

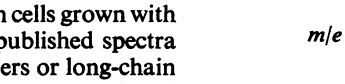
Optical rotation

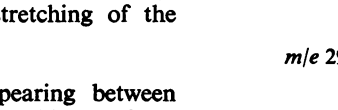
The unknown compound (from cells grown with palmitic acid) was found to have a specific rotation $[\alpha]_D^{25}$ +6.49° (c 0.77 in chloroform), equivalent to a molecular rotation of $[M]_D^{25}$ 34.9.

Mass spectroscopy

The mass spectrum of the unknown compound (dicarboxylic acid dimethyl ester) obtained from palmitic acid-grown bacteria gave a molecular ion with the formula $C_{34}H_{66}O_4$. It did not change when the compound was treated with acetyl chloride or a silylating reagent (trimethylsilylimidazole), indicating the absence of hydroxy groups. The compound was saponified by alkaline hydrolysis [6% (w/v) KOH in aq. 95% (v/v) ethanol; 1h under reflux], and the free acid produced was remethylated (diazomethane or methanolic HCl) to regenerate an ester that ran identically with the original compound on t.l.c. This suggested that the original acid contained at least one carboxylic acid grouping. Re-esterification of the free acid in the presence of [^{2}H]methanol alone or [^{2}H]methanol/methanol (1:1, v/v) mixture instead of methanol produced an increase of 6 in the molecular ion (Table 1) and a characteristic 1:2:1 triplet with the mixed reagent, showing that the compound contained two methylated carboxylic acid groups.

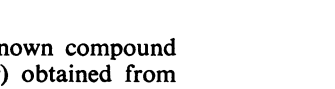
The 70eV mass spectrum of the dicarboxylic acid dimethyl ester derived from palmitic acid-grown cells (Fig. 1) was characterized by intense ions at *m/e* 74 and 87 as well as a series of ions of the general type CH_3–O–CO–$[CH_2]_n^+$, i.e. *m/e* 143 ($n=6$), suggesting a saturated methyl ester. This series of ions continued up to and included two particularly intense clusters of ions 28 mass units apart centred at *m/e* 269 ($n=15$) and *m/e* 297 ($n=17$) (Table 1), indicative of the presence of a fragmentation-directing functional group such as a branching point. On the basis of the evidence available from accurate mass measurements, deuterium labelling and the examination of homologous series, possible structures for these ions are:

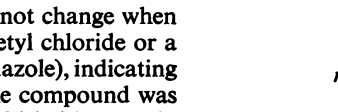
m/e 269  (I)

m/e 297  (II)

The less-intense ion at *m/e* 241 ($n=13$) represents cleavage distal to the second substituted carbon atom.

Two major groups of ions centred at *m/e* 238 and 266 were also apparent. These ions differed from the groups at *m/e* 269 and 297 in that they contained only one oxygen atom, not two, and were not shifted by deuteration of the ester methoxy group (Table 1). The most likely structure for the ions at *m/e* 238 and 266 was that of acylium ion counterparts of the ions at *m/e* 269 (I) and 297 (II) produced by formal loss of the methoxy group, and of the general formula $[CH_2]_n$–CO^+:

m/e 238  (III)

m/e 266  (IV)

An ion at *m/e* 210 ($C_{14}H_{26}O$; mol.wt. 210.2003) is the counterpart of that at *m/e* 241. Ions at *m/e* 219 ($C_{16}H_{27}$; mol.wt. 219.2132) and *m/e* 227 ($C_{14}H_{27}O_2$; mol.wt. 227.2014; one CH_3O group) represent further fragmentation from the carbonyl and/or tertiary carbon atoms respectively.

When the bacterium is grown with fatty acids other than palmitic acid, the mass spectra of the methyl esters of the dicarboxylic acids isolated show homologous behaviour predictable on the basis of condensation of two fatty acid molecules (Table 2). An unusual feature of the spectra of this class of

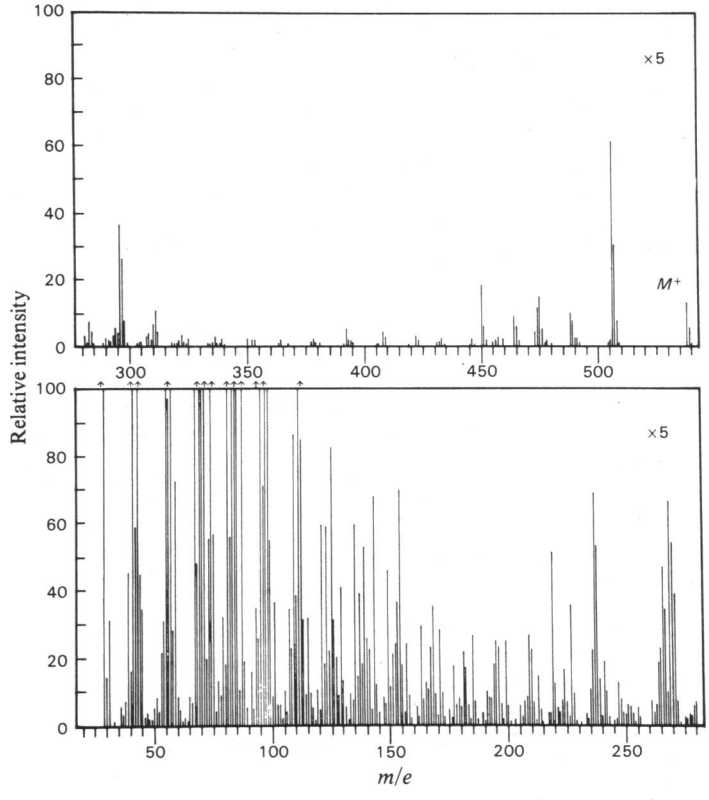


Fig. 1. *70eV mass spectrum of the compound (dicarboxylic acid dimethyl ester) from cells of Butyrivibrio S2 grown in the presence of palmitic acid*

For details of culture and preparation of the dicarboxylic acid dimethyl ester see the Materials and Methods section.

Table 1. *Diagnostic ions in the mass spectrum of the compound (dicarboxylic acid dimethyl ester) obtained from cells of Butyrivibrio S2 cultured in the presence of palmitic acid*

Group	*m/e*	Intensity (%)	Mass increment	Composition	$10^3 \times$ Deviation (mass units)	C^2H_3O	Shift C^2H_3O/CH_3O
A	538 (M^+)	2.6	0.4948	$C_{34}H_{66}O_4$	(−1.3)	544	544 541 } (1:2:1) 538
B	506	12.3	0.4724	$C_{33}H_{62}O_3$	(2.5)	509	509 506 } (1:1)
C	475	3.0	0.4538	$C_{32}H_{59}O_2$	(2.3)	475	475
	474	2.3	0.4462	$C_{32}H_{58}O_2$	(2.6)	474	474
	450					453	453/450
D	297	5.2	0.2792	$C_{19}H_{37}O_2$	(−0.2)	300	300/297
	296	7.3	0.2710	$C_{19}H_{36}O_2$	(−0.5)	299	299/296
E	271	7.8	0.2612	$C_{17}H_{35}O_2$	(−2.5)	274	274/271
	270	10.8	0.2552	$C_{17}H_{34}O_2$	(−0.7)	273	272/270
	269	13.3	0.2452	$C_{17}H_{33}O_2$	(−2.8)	272	272/269
F	267	6.8	0.2336	$C_{17}H_{31}O_2$	(1.2)	270	270/267
	266	9.3	0.2568	$C_{18}H_{34}O$	(−4.1)	266	266
	265	4.5	0.2539	$C_{18}H_{33}O$	(0.8)	265	265
	264	3.7	0.2484	$C_{18}H_{32}O$	(3.1)	264	264
G	238	10.6	0.2289	$C_{16}H_{30}O$	(−0.7)	238	238
	237	13.7	0.2213	$C_{16}H_{29}O$	(−0.6)	237	237
	236	4.4	0.2100	$C_{16}H_{28}O$	(−4.0)	236	236

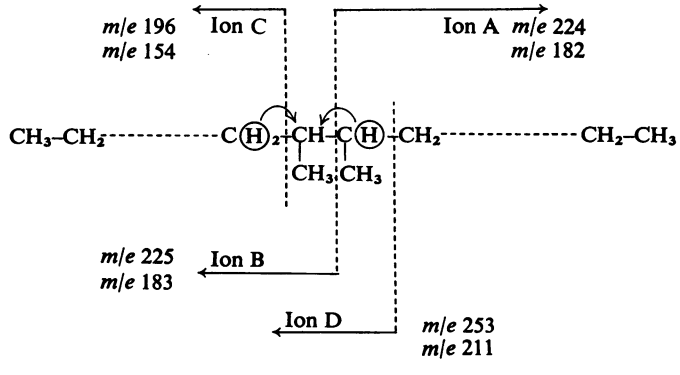
Table 2. *Masses of the major ions in each group for the compounds (dicarboxylic acid dimethyl esters) obtained from cells of Butyrivibrio S2 cultured in the presence of different fatty acids*

Precursor acid	Group ...	*m/e* A (M^+)	B	C	D	E	F	G
Tetradecanoic ($C_{14:0}$)		482	450	419*	269	243	239	210
				418	268*	242	238	209*
						241*	237	
Pentadecanoic ($C_{15:0}$)		510	478	447*	283	257	253	224
				446	282*	256	252*	223*
						255*	251	
Hexadecanoic ($C_{16:0}$)		538	506	475*	297	271	267	238
				474	296*	270	266*	237*
						269*	265	
Heptadecanoic ($C_{17:0}$)		566	538	503*	311	285	281	252
				502	310*	284	280*	251*
						283*	279	
Octadecanoic ($C_{18:0}$)		594	562	531*	325	299	295	266
				530	324*	298	294	265*
						297*	293	
Octadec-*trans*-11-enoic ($C_{18:1}$)		590	558	527	322	—	295	— †
				526*	321*	—	294	— †
						—	293*	†
							292	†
							291	†

* Most intense ion in any particular group.
† Characteristic fragmentation pattern lost below *m/e* 400.

compounds is the marked formal loss of the elements of methanol from the molecular ion (i.e. for dicarboxylic acid dimethyl ester from palmitic acid-grown bacteria *m/e* 538→506), followed by a second loss of a methoxy group or methanol (*m/e* 506→475, *m/e* 506→474). A $(C_{11:0})_2$ homologue of the unknown compound has been prepared by chemical synthesis (R. A. Klein, unpublished work), and the mass spectrum shows that the same fragmentation processes occur under electron impact. Metastable transition studies have substantiated the formal loss of methanol from the molecular ion of dicarboxylic acid dimethyl ester, and we have shown that this fragmentation is a notable feature of the mass spectrum of the totally synthetic $(C_{11:0})_2$ homologue.

The 70eV mass spectrum of the C_{32} hydrocarbon prepared from dicarboxylic acid dimethyl ester (Fig. 2) was compared with that of the C_{26} synthetic hydrocarbon. The spectra are dominated by cleavage and rearrangement processes associated with the tertiary carbon atoms, with relatively inconspicuous molecular ions:



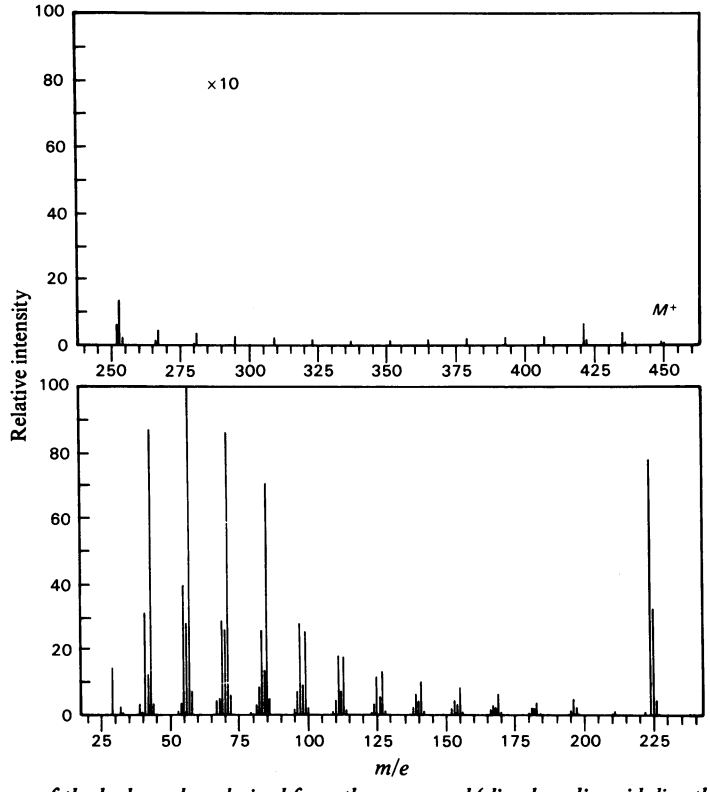


Fig. 2. *70eV mass spectrum of the hydrocarbon derived from the compound (dicarboxylic acid dimethyl ester) that had been prepared from cells of Butyrivibrio S2 grown in the presence of palmitic acid*
For details see the Materials and Methods section.

Accurate mass measurements confirmed the structure of the two molecular ions $C_{32}H_{66}$ (measured *m/e* 450.5168; calculated *m/e* 450.5164) and $C_{26}H_{54}$ (measured *m/e* 366.4226; calculated *m/e* 366.4225). The composition of ions A–D was also established by high-resolution mass determination. The most abundant high-mass ion in the spectrum of both the C_{26} hydrocarbon and the C_{32} hydrocarbon (ion A) results from cleavage between the two tertiary carbon atoms with the rearrangement of one hydrogen atom (*m/e* 182 and *m/e* 224 respectively).

^1H n.m.r. spectroscopy

The spectrum contained resonances ascribable to ester methoxy groups (CH_3–O–; 3.65δ, singlet), methylene groups α to a carbonyl group (–CH_2–CO–; 2.27δ, triplet, J_{HH} = 7Hz), intrachain methylene and methine groups (–CH_2– and CH; 1.28δ, singlet) as an envelope with a low-field shoulder (1.5–1.8δ), and terminal methyl groups (CH_3–; 0.75δ, doublet, J_{HH} = 6Hz) resonating at higher field than the isopropyl methyl group protons in, for example, 13-methyltetradecanoic acid (0.88δ, doublet, J_{HH} = 6Hz).

Integration of the ^1H n.m.r. spectrum gave the following values for the individual peak areas in arbitrary units: CH_3–, 13; CH_3O–, 16; –CH_2–CO–, 11; (CH_2+CH), 132. The possible molecular formula $C_{34}H_{66}O_4$ indicated by mass spectrometry taken in conjunction with the n.m.r. data suggested that the compound was the dimethyl ester of a saturated dicarboxylic acid containing no rings.

The degree of branching was calculated according to the general procedure of Sonneveld *et al.* (1962) from the peak areas, and gave a value of 1.68, i.e. two branching points. The two methyl groups are magnetically equivalent, suggesting either near-neighbour methyl groups or the presence of an isopropyl group.

Part of the 100MHz ^1H n.m.r. spectrum for the unknown compound (dicarboxylic acid dimethyl ester), showing the region of the methylene envelope and terminal methyl signals, is reproduced in Fig. 3. The doublet at 0.75δ (J = 6.5Hz) assigned to the

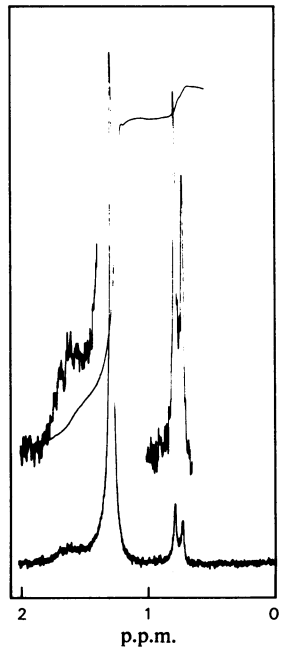


Fig. 3. *100 MHz ^1H n.m.r. spectrum of the compound (dicarboxylic acid dimethyl ester) from cells of Butyrivibrio S2 grown in the presence of palmitic acid*
For details of culture and preparation of the dicarboxylic acid dimethyl ester see the Materials and Methods section.

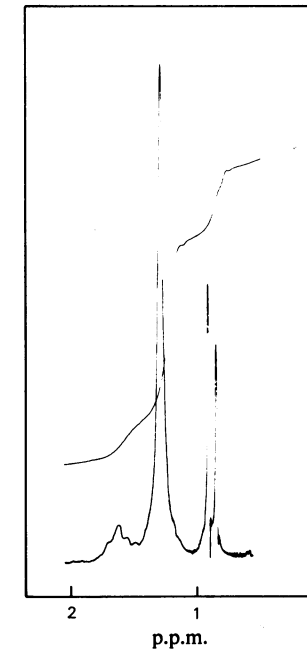


Fig. 4. *100 MHz ^1H n.m.r. spectrum of 13-methyltetradecanoic acid*
For details see the Materials and Methods section.

chain methyl groups occurs at relatively high field compared with, for example, that of the terminal isopropyl group in 13-methyltetradecanoate (0.85δ) shown in Fig. 4. Examination of the spectrum for the optically active C_{32} hydrocarbon prepared from the unknown compound (Fig. 5) shows a high-field methyl doublet ($J = 6.3$ Hz) as well as an apparent singlet at 0.90δ. This singlet is in fact a triplet corresponding to the terminal (CH_3–CH_2) methyl group. Such methyl triplets are often poorly resolved in hydrocarbons, with indistinct shoulders (cf. this region of the spectrum for 2-bromotridecane shown in Fig. 6).

Examination of the spectrum for the synthetic C_{26} hydrocarbon (Fig. 7) indicates a more complex situation. The (CH_3–CH_2) methyl triplet is superimposed on an apparent 1:2:1 triplet at higher field. We interpret this high-field triplet as being composed of two doublets with their resonances fortuitously separated by the coupling constant of approx. 6 Hz, with the doublet at lower field representing the *meso* isomer and the one at higher field the racemic isomer, as seen in the optically active material obtained from biological sources. These assignments are in keeping with the results obtained by ^{13}C n.m.r. as discussed below.

^{13}C n.m.r. spectroscopy

Twelve resonances were observed in the ^{13}C n.m.r. spectrum of the unknown compound (dicarboxylic acid dimethyl ester) from bacteria grown with palmitic acid, suggesting either a very high degree of accidental magnetic equivalence or considerable molecular symmetry in a molecule of formula $C_{34}H_{66}O_4$. Moreover, the ester carbonyl (174.46δ) and methoxy (51.42δ) and the terminal methyl (14.48δ) resonances were present as singlets, indicating magnetic equivalence and hence molecular symmetry.

On the basis of Lindeman–Adams parameters (see Klein & Kemp, 1977) and published values for methyl esters of saturated fatty acids (see, e.g., Gunstone *et al.*, 1977) the following structure is proposed:

$$CH_3\text{–}O\text{–}CO\text{–}[CH_2]_{13}\text{–}\underset{\displaystyle CH_3}{\underset{|}{CH}} - \underset{\displaystyle CH_3}{\underset{|}{CH}}\text{–}[CH_2]_{13}\text{–}CO\text{–}O\text{–}CH_3$$

Experimentally determined chemical shifts and the predicted values are shown in Table 3.

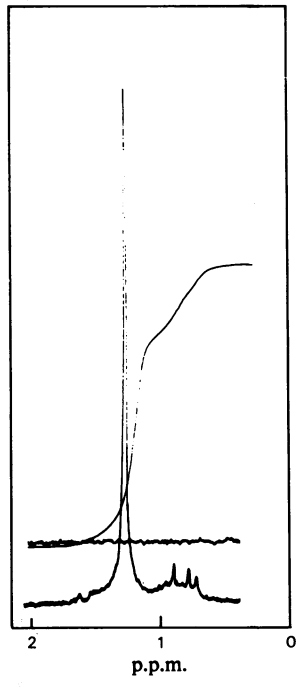


Fig. 5. 100 *MHz* ^1H *n.m.r. spectrum of the hydrocarbon derived from the compound (dicarboxylic acid dimethyl ester) that had been prepared from cells of Butyrivibrio S2 grown in the presence of palmitic acid*
For details see the Materials and Methods section.

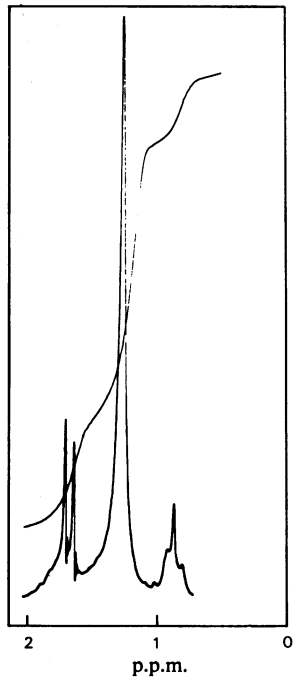


Fig. 6. 100 *MHz* ^1H *n.m.r. spectrum of 2-bromotridecane*
For details see the Materials and Methods section.

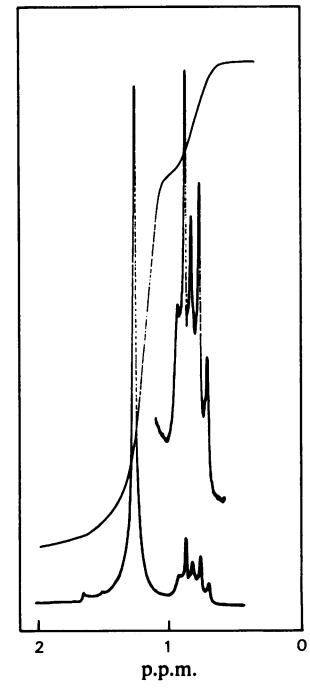


Fig. 7. 100 *MHz* ^1H *n.m.r. spectrum of* 12,13-*dimethyltetracosane*
For details of preparation see the Materials and Methods section.

Table 3. *Assignments in the* ^{13}C *n.m.r. spectrum of the compound (dicarboxylic acid dimethyl ester) obtained from cells of Butyrivibrio S2 cultured in the presence of palmitic acid*

The values for the predicted shifts were: [a] from Gunstone *et al.* (1977) for long-chain 9,11-dioxo acids; [b] by comparison with methyl myristate (Johnson & Jankowski, 1972); [c] calculated by using Lindeman–Adams substitution parameters.

Observed shift (p.p.m.)	Assignment	Predicted shift (p.p.m.)
51.42	CH_3–0–	51.36[a]; 51.2[b]
174.46	C-1 (C=0)	174.25[a]; 174.0[b]
34.19	C-2	34.14[a]; 34.1[b]
25.03	C-3	24.98[a]; 25.0[b]
29.31	C-4	29.39[a]; 29.4[b]
29.73	C-5–C-11	29.75[a]; 29.7[b]
30.09	C-12	30.21[c]
27.78	C-13	27.52[c]
35.03	C-14	34.22[c]
36.72	C-15 (CH_3–*C*H)	37.03[c]
14.49	*C*H_3–CH	16.64[c]

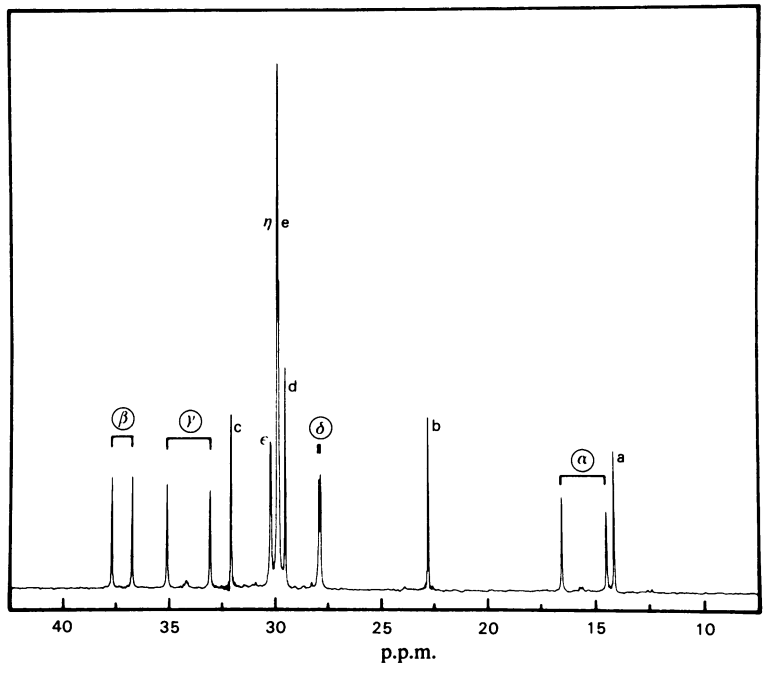
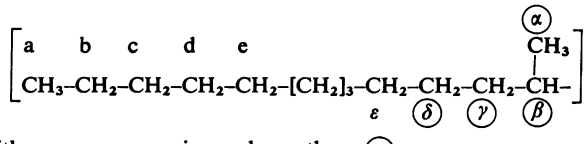


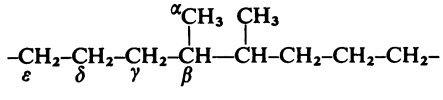
Fig. 8. ^{13}C *n.m.r. spectrum of* 12,13-*dimethyltetracosane*

For details of preparation see the Materials and Methods section. Carbon atoms are shown as follows:



Those distinguishable as either *meso* or racemic are shown thus: (α).

Table 4. *Assignment of doublets in* ^{13}C *n.m.r. spectrum of* 12,13-*dimethyltetracosane prepared from* 2-*bromotridecane*

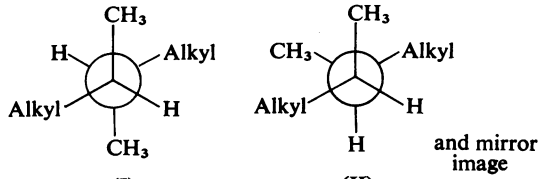


Carbon atom ...	Chemical shift (p.p.m.) α	β	γ	δ	ε
$(C_{16:0})_2$ diabolic acid	14.49	36.72	35.03	27.78	30.09
C_{26} hydrocarbon					
racemic	14.53	36.83	35.20	27.96	30.30
meso	16.58	37.80	33.16	28.03	30.30
Calculated by Lindeman–Adams parameters	16.64	37.03	34.22	27.52	30.21

Substitution parameters give rise to noticeable errors in the presence of branching, and the relatively high-field shift for the methyl carbon atoms may be explained in terms of the steric compression shift (Grant & Cheney, 1967; Levy & Nelson, 1972). The chemical shifts for the methyl group protons should be compared with those of 7,9-dimethyldec-1-ene or of α-tocopherol or the sterically hindered methyl groups in cyclopentanophenanthrene derivatives (Bhacca *et al.*, 1962).

The ^{13}C n.m.r. spectrum of the synthetic C_{26} hydrocarbon gave doublets corresponding to carbon atoms in the neighbourhood of the vicinal dimethyl group (Fig. 8), each doublet being made up of a resonance assigned to either the *meso* or the racemic isomer. By comparison with the optically active dicarboxylic acid dimethyl ester it was possible to assign these doublets as shown in Table 4.

It is noteworthy that, as might be expected, the calculation of the chemical shift by using Lindeman–Adams parameters for carbon atoms near to a branching point is more reliable for the less sterically compressed *meso* isomer. The steric compression shift of the racemic methyl resonance to higher field is probably a reflection of the higher probability of a *gauche* environment for these methyl groups, as indicated in the projections (I) and (II) shown below:



(I) *meso* (II) racemic

Similar assignments have been advanced by Carman *et al.* (1971) in their discussion of the ^{13}C n.m.r. spectra of the racemic and *meso* isomers of 2,3-dichlorobutane and 2,4-dichloropentane.

The presence of the vicinal dimethyl substituents has a profound effect on the g.l.c. retention time. The decrement in the retention index on going from the C_{26} n-alkane to the 12,13-dimethyltetracosane is -94 ± 2, whereas the difference between the straight-chain dimethyl 1,22-dioate and the dimethyl ester of $(C_{11:0})_2$ diabolic acid (R. A. Klein, unpublished work) is -93 ± 10.

The proposed structure has received support from the results of oxidation experiments with CrO_3 in acetic acid. A homologous series of normal dicarboxylic acids, identified by combined g.l.c. and mass spectrometry, was obtained up to and including tetradecanedioic acid, which represented the major oxidative cleavage product. As might be expected from the suggested structure, a small but significant amount of pentadecanedioic acid was also detected, before the homologous series of dicarboxylic acids was interrupted.

At this stage there is little positive information on the relative conformation of the alkyl chains, although the spectroscopic evidence tends to favour the *trans* conformation in dilute solution. The possibility exists, however, that the alkyl chain may be in the *cis* conformation in the solid or liquid state given the rather low melting point (42–43°C) for the compounds' chain length. Whether diabolic acids exist as straight or 'hairpin' molecules may have some relevance in considering their possible role in membrane structure. Appropriate monolayer studies may provide further insight into the conformation of the natural form.

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