Isolation and Identification of Cyclopropane Fatty Acids from the Millipede *Graphidostreptus tumuliporus* (Karsch) (Myriapoda:Diplopoda)*

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ABSTRACT: The isolation of three important unusual fatty acids from the lipids of the millipede *Graphidostreptus tumuliporus* was undertaken. The purification of these components was achieved by urea fractionation of the saturated fatty acid methyl ester fraction, followed by preparative gas chromatography. The absorptions at δ −0.26 and +0.64 in the nuclear magnetic resonance spectra and the absorption bands at 1020 and 3059 cm^{-1} in the infrared spectra immediately demonstrated the presence of a cyclopropane group in all three components. The molecular weights of these compounds were determined by mass spectrometry, revealing a total number of carbon atoms of 17, 18, and 19, respectively. According to all data obtained, these components were identified as *cis*-C_{17}-, *cis*-C_{18}-, and *cis*-C_{19}-cyclopropane fatty acids. Not only the occurrence of cyclopropane fatty acids in a millipede is a most interesting feature, but also the structure of the most abundant component (18:0 cyclo), because until now only odd-numbered cyclopropane fatty acids have been reported. The metabolic activity of these fatty acids in *G. tumuliporus* was demonstrated by their labeling after injection of the animals with [1-^{14}C]acetate.

Recently we have reported the occurrence of three unusual saturated fatty acids in the millipede *G. tumuliporus* (Karsch). These components accounted for nearly 30% of the fatty acid content of the total lipids. At that time the structure of these fatty acids was not further investigated, but on account of their gas chromatographic behavior on two different stationary phases it was suggested that their structure might be a multiple-branched one (Oudejans *et al.*, 1971).

In this paper we describe the isolation of these fatty acids by means of column chromatography, urea fractionation, and preparative gas chromatography and their identification using analytical gas chromatography, infrared, nuclear magnetic resonance, and mass spectrometry.

Material and Methods

Twenty-four female specimens (total fresh weight 294.0 g) of an African millipede, *Graphidostreptus tumuliporus* (Karsch), were received from the Institut Fondamental d'Afrique Noire, Dakar, Senegal.

Each of the animals was injected with 10 μCi of [1-^{14}C]-acetate (New England Nuclear, specific activity 2 mCi/mmole). Incubation without supply of food was ended after 96 hr by freezing the animals at −26°.

Lipids were extracted and the fatty acids (10.64 g) were isolated as described before (Oudejans *et al.*, 1971). Samples of the fatty acid fraction were methylated with diazomethane (Schlenk and Gellerman, 1960) and separated from non-fatty acid methyl esters on a silicic acid column (Mallinckrodt, 100 mesh) with hexane–ether (95:5, v/v). From 1.164 g of the fatty acid methyl esters the saturated fraction (0.504 g) was obtained by mercury adduction according to Goldfine and Bloch (1961). This fraction contained the three fatty acids under investigation (named A, B, and C).

Urea Fractionation. The saturated fatty acid methyl esters (0.500 g) and urea (0.5 g) were dissolved in 8 ml of methanol at 70° and cooled to room temperature under stirring during the first 30 min. After 3 hr the white crystalline complex containing most of the straight-chain fatty acid methyl esters was removed. The filtrate was diluted with methanol and acidified with 1% hydrochloric acid, after which the fatty acid methyl esters (mostly with a branched chain) were extracted with hexane.

After repeating this complexing procedure two times, a fraction of 40.6 mg was obtained containing over 93% branched-chain-type components, mostly A, B, and C, but some traces of isobranched ones were still present (Figure 1-II). A fraction with an even higher content of branched-chain components (>99%) was obtained by chromatography of another sample of the saturated fraction on a column containing urea beads in methanol saturated with urea (Figure 1-III). However, the yield was lower than in the former complexing procedure.

Gas Chromatography. Analytical gas–liquid chromatography (glc) was performed on a Becker instrument, type 1452, with dual-flame ionization detection. Two glass columns of 1.80 m × 3.8 mm i.d. were used, packed with 20% polyethylene glycol adipate (PEGA)[1] + 3% phosphoric acid, and 10% Apiezon L, respectively, on acid-washed Chromosorb W (60–80 mesh); column temperature 180°, outlet flow of carrier gas (nitrogen) 50 cc/min. The purity of all fractions during the whole fractionating procedure of the fatty acids was checked on both stationary phases.

Preparative glc was carried out on a Becker instrument type 1438E-1458K, equipped with katharometer detection. A stainless steel column (3.00 m × 6 mm i.d.) was used, packed with 20% PEGA on acid-washed Chromosorb W (60–80 mesh); column temperature 182°, outlet flow of carrier gas (helium)

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[1] Abbreviation used: PEGA, polyethylene glycol adipate.

TABLE I: Weights and Radioactivities of Components A, B, and C after Preparative Gas Chromatography.

Component	Wt (mg)	Radioactivity (dpm/mg)
Starting material	34.78	3891
A	1.04	*a*
B	12.03	3685
C	7.73	4361
Rest	6.37	*a*
Recovery	78.12%	

[a] Not determined.

100 cc/min. The separated fatty acid methyl esters were collected on glass beads (0.45–0.50 mm diameter).

Infrared (ir) Spectrometry. Ir spectra of the fatty acid methyl esters were recorded on a Perkin-Elmer 457 spectrophotometer. Free films of the neat liquids between a pair of NaCl windows were used.

Nmr Spectrometry. Nmr spectra of 100 MHz were recorded on a Varian XL-100/15 spectrometer. Single-scan spectra of solutions of 5–10 mg of the fatty acid methyl esters in 0.3 ml of CCl_4 were obtained locking on tetramethylsilane as an internal reference (δ values in parts per million). For recording the region below δ 0.3, the deuterium-lock mode was applied, using hexadeuteriobenzene as a solvent. In the latter case the nondeuterated fraction of the hexadeuteriobenzene was used as an internal reference (δ 7.15 from tetramethylsilane).

Mass Spectrometry. Mass spectra of 70 eV were recorded with an AEI MS-9 mass spectrometer.

Radioactivity. Radioactivities of all fractions were measured in toluene–Omnifluor (New England Nuclear) with a Packard liquid scintillation spectrometer, Model 2420.

Results and Discussion

After urea fractionation of the saturated fatty acid methyl esters, a fraction containing almost exclusively branched-chain components was obtained with the following composition (in per cent): A, 7.3; B, 51.8; C, 30.8; other branched components, 3.7; straight chain, 6.4. A gas chromatogram of this mixture is represented in Figure 1. Considering the fact that A, B, and C are members of a homologous series (Oudejans *et al.*, 1971) and amount to nearly 90% of this fraction, ir and nmr spectra of the total mixture were recorded.

In the nmr spectra (in hexadeuteriobenzene) the two absorptions at δ −0.26 and +0.64 immediately revealed the presence of a cyclopropane group in the main components (Christie *et al.*, 1968). Other types of branching in components A, B, and C were absent.

The ir absorption bands at 1020 and 3059 cm^{-1} which are characteristic for 1,2-disubstituted cyclopropanes (Dijkstra and Duin, 1955; MacFarlane *et al.*, 1957; Kaneshiro and Marr, 1961; Christie *et al.*, 1968) confirmed the presence of a cyclopropane system in the fatty acid methyl esters. In order to determine the structure of each individual component preparative gas chromatography was carried out on 34.78 mg of the mixture. In this way pure components A, B, and C (purity >99%) were obtained as checked by analytical gas chromatography. The weights and specific radioactivities of A, B, and C are summarized in Table I.

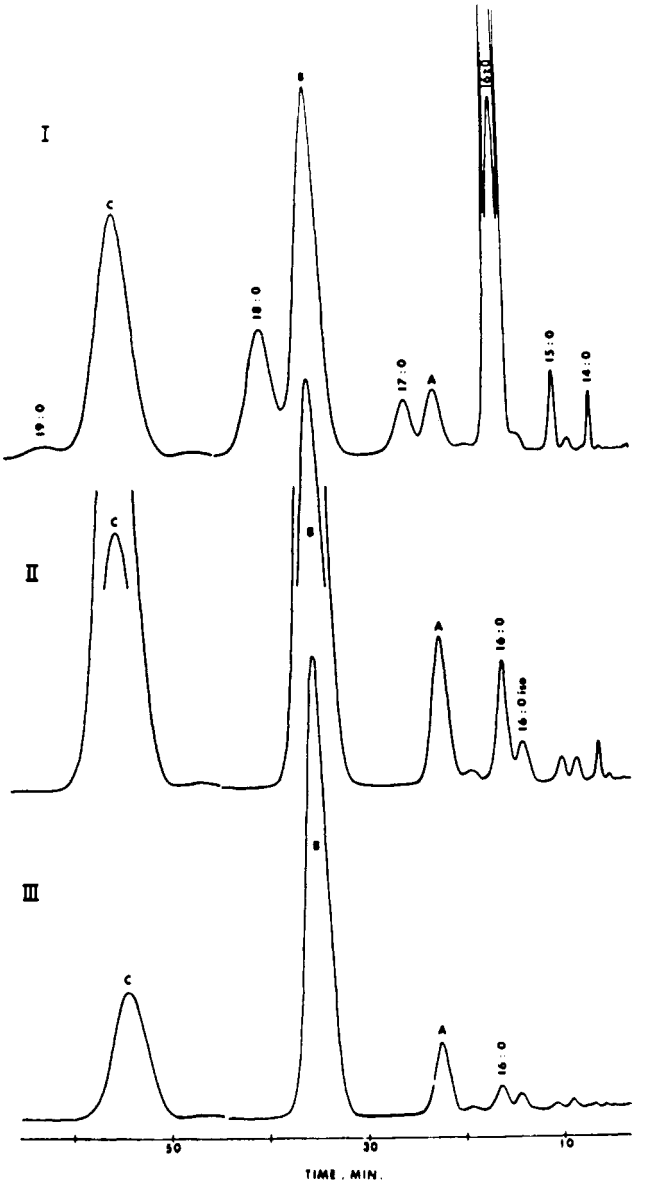
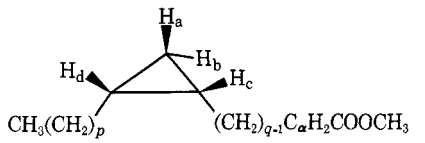


FIGURE 1: Part of the gas chromatogram on a 10% Apiezon L column of: (I, total saturated fatty acid methyl ester fraction; (II, *idem*, after fractionation with urea (three times); (III, *idem*, after column chromatography on urea beads.

Spectroscopic Properties of Components A, B, and C. INFRARED. The three compounds all gave the specific cyclopropane absorption bands at 1020 and 3059 cm^{-1} (neat liquids). Figure 2 represents the infrared spectrum of component B.

NUCLEAR MAGNETIC RESONANCE. The nmr spectra of A, B, and C were very similar. The absorptions at δ −0.26 (one proton, H_b) and at δ +0.64 (three protons, H_a, H_c, and H_d) are consistent with a cis arrangement of the two alkyl substituents on the cyclopropane ring. The chemical shifts of the



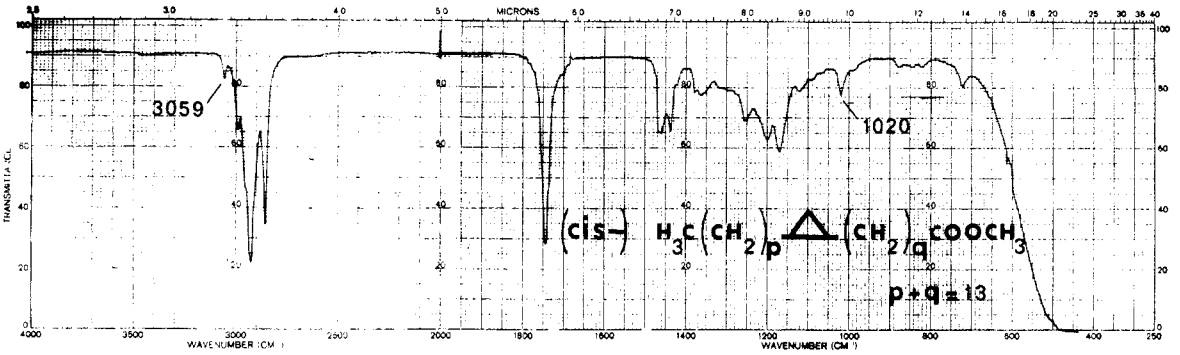


FIGURE 2: Infrared spectrum of component B.

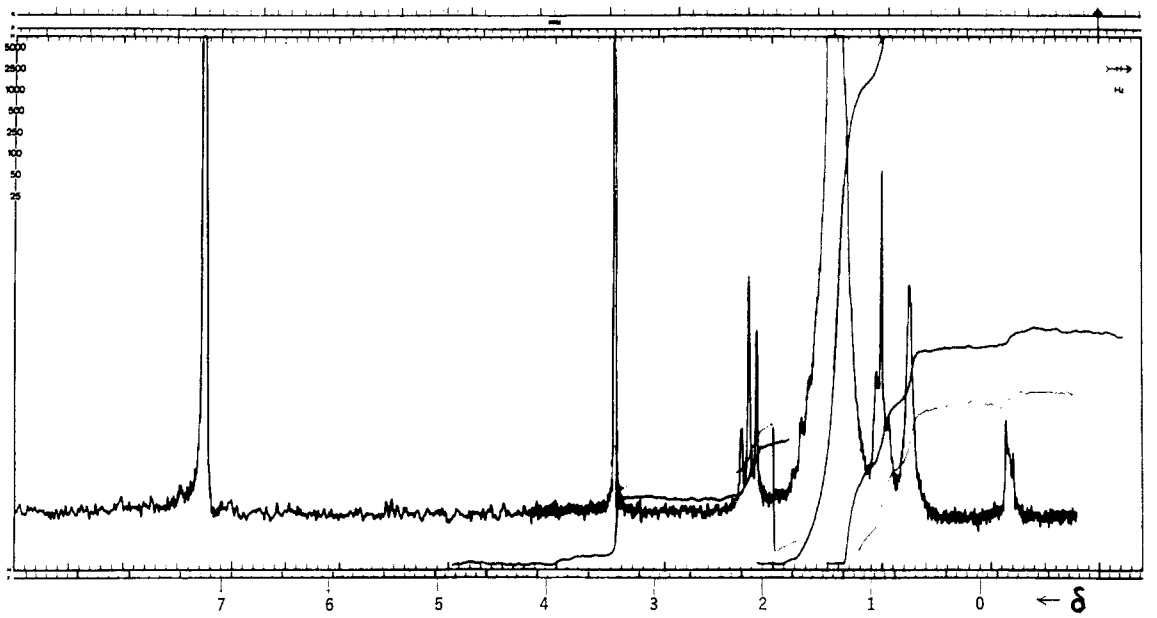


FIGURE 3: 100-MHz nmr spectrum of component B in C_6D_6.

TABLE II: Principal Features in the Nuclear Magnetic Resonance Spectra of Compounds A, B, and C.[a]

Protons	In CCl_4	In C_6D_6
OCH_3	3.59 (s, 3 H)[b]	3.36 (s, 3 H)
$C_\alpha H_2$	2.22 (t, 2 H)	2.12 (t, 2 H)
$(CH_2)_p$, $(CH_2)_{q-1}$	1.28 (b, 2($p + q - 1$) H)	1.32 (b, 2($p + q - 1$) H)
CH_3	0.88 (t, 3 H)	0.90 (t, 3 H)
$H_a + H_c + H_d$	0.58 (b, 3 H)	0.64 (b, 3 H)
H_b	?	−0.26 (m, 1 H)

[a] Chemical shifts are expressed in parts per million down field from internal tetramethylsilane ($\delta = 0$). [b] s = singlet; t = triplet; b = broad band; m = multiplet.

cyclopropane protons indicate that the position of the cyclopropane ring is somewhere in the middle of the aliphatic chain (Christie *et al.*, 1968; Christie, 1970). Table II lists the chemical shift data of A, B, and C and Figure 3 represents the nmr spectrum of B in hexadeuteriobenzene.

MASS SPECTROMETRY. The mass spectra show the peaks characteristic for a fatty acid methyl ester, for example, M^+, $M - 31$, 74. Apart from the decomposition pattern of an aliphatic chain no other characteristic fragments are present. The molecular weights of A, B, and C are according to their mass spectra:

A	282	$H_3C(CH_2)_p$—△—$(CH_2)_qCOOCH_3$	$p + q = 12$
B	296		$p + q = 13$
C	310		$p + q = 14$

Unfortunately the position of the cyclopropane ring cannot be determined directly by mass spectrometry (Pohl *et al.*, 1963; Wood and Reiser, 1965; Christie and Holman, 1966). The exact location of this group will be investigated in the near future according to McCloskey and Law (1967).

Gas Chromatography. Relative retention times on the stationary phases PEGA and Apiezon L of components A, B, and C have already been given in our first paper (Oudejans *et al.*, 1971).

The presence of cyclopropane fatty acids in female specimens of *G. tumuliporus* is a unique feature because until now this type of fatty acids has been reported only in bacteria, plants, and protozoa.

Moreover, the number of carbon atoms of the most abundant component (18 carbon atoms) is different from the cyclopropane fatty acid structures published already, which are all odd numbered (review by Christie, 1970). Both the high amount of these fatty acids and their radioactivities after injection of [1-^{14}C]acetate indicate that these substances play an important role in the metabolism of this millipede, which is discussed in another paper (van der Horst *et al.*, 1971).

Acknowledgments

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Effects of Estradiol on Uterine Ribonucleic Acid Metabolism. I. *In Vitro* Uptake and Incorporation of Ribonucleic Acid Precursors*

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ABSTRACT: The *in vitro* uptake and incorporation of [5-^{3}H]-uridine, [^{32}P]P_i, and L-[*methyl*-^{3}H]methionine were monitored at intervals during a 2-hr incubation to assess RNA synthesis in uteri of immature rats at various times following intraperitoneal injection of 17β-estradiol. With short *in vitro* pulses, uptake and incorporation of uridine were most prominent during the first few hours after estrogen treatment; with P_i, increases were not evident even at 12 hr; while with methionine the increases were progressive throughout this period. During longer *in vitro* pulses the rate of incorporation of uridine fell to control levels and even lower, particularly, after longer periods of hormone treatment; that of P_i was somewhat enhanced; while that of methionine increased linearly throughout the period of incubation. These data are interpreted on the basis of the effects of estradiol on parameters influencing the specific activities of the precursor pools in question, as well as on the rate of synthesis of RNA.

While the marked accumulation of uterine RNA accompanying the estrogen response has been well documented, the use of a variety of isotopic precursors, as well as labeling systems, to monitor the rate of uterine RNA synthesis has yielded discordant results. For example, Gorski and Nicollete (1963), using a 60-min pulse *in vivo*, observed significantly increased [^{32}P]P_i incorporation into RNA of uterine subcellular fractions during the initial hours of the estrogen response. In an analogous *in vitro* labeling system, increased incorporation was not detected. On the other hand, Billing *et al.* (1969b), utilizing an *in vivo* system in which the radioactivity associated with the adenine nucleotide pool was stabilized, reported that incorporation of this isotope into uterine RNA increased only slightly during the initial phase of the response and did not become substantial until after 5 hr. In a somewhat different type of study, Hamilton *et al.* (1968) found that during a 10-min pulse *in vivo*, [5-^{3}H]uridine incorporation into uterine nuclear RNA was maximal 20 min after estrogen administration.

While it is recognized that the extent of incorporation of an

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