Identification of Cyclopentenyl Fatty Acids by ^{1}H and ^{13}C Nuclear Magnetic Resonance

Pierre Blaise, Marie Farines*, and Jacques Soulier

Laboratoire de Chimie Organique des Substances Naturelles, Université F, 66860 Perpignan Cedex, France

ABSTRACT: Gorlic, chaulmoogric and hydnocarpic fatty acids, specific to the seed oil of the genus *Hydnocarpus* sp. (Flacourtiaceae), are determined only with difficulty by gas chromatography. These fatty acids were isolated in their methyl ester form by a combination of different chromatographic techniques (thin-layer chromatography/Ag^{+} and high-pressure liquid chromatography). The proton and carbon nuclear magnetic resonance analysis of these fatty acid methyl esters showed some characteristic signals of the cyclopentenyl ring. The presence of these signals in the proton and/or carbon nuclear magnetic resonance spectrum of an oil thus will allow us to confirm the presence of these cyclopentenyl fatty acids in lipids.
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KEY WORDS: Chaulmoogric acid, Flacourtiaceae, gorlic acid, ^{1}H and ^{13}C NMR, HPLC, hydnocarpic acid, *Hydnocarpus* seed oil, identification, TLC/Ag^{+}, 2D NMR.

The seed oils of many species of plants of the Flacourtiaceae family are known to contain fatty acids with a terminal cyclopentenyl group (1,2). These cyclopentenyl fatty acids constitute up to 80% of the total fatty acids of the seed oils of *Hydnocarpus,* which have been used in the treatment of leprosy (3). Identification of these compounds by gas–liquid chromatography (GLC) is not straightforward because they can be confused, by calculation of their equivalent chain-length, with polyunsaturated fatty acids. These compounds have been identified by GLC/mass spectroscopy after derivatization of the fatty acids (4–8). The ^{13}C nuclear magnetic resonance (NMR) study of many fatty acid methyl esters (FAME) with particular structures has been carried out by Gunstone (9,10). As far as we know, there have been no NMR studies on cyclopentenyl fatty acids. The various fatty acids of *Hydnocarpus* seed oils were fractionated by silver nitrate thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC); a ^{1}H and ^{13}C NMR study with $^{1}H/^{1}H$, $^{1}H/^{13}C$ and heteronuclear multiple bond correlation (HMBC) bidimensional techniques made it possible to differentiate the spectral characteristics of these compounds. NMR study of the oil easily shows the presence of these fatty acids.

*To whom correspondence should be addressed at Laboratoire de Chimie Organique des Substances Naturelles, Avenue de Villeneuve, Université F, 66860 Perpignan Cedex, France. E-mail: farines@univ-perp.fr.

MATERIALS AND METHODS

Oil (50 mg) was transesterified with methanol in the presence of sodium methylate (11). The FAME were analyzed by GLC on a DI 200 chromatograph (Unicam, Argenteuil, France), fitted with a glass needle evaporator injector (Ross type) and a flame-ionization detector. The column (25 m long, 0.32 mm i.d.) was coated with BPX70 (film thickness, 0.25 μm) (SGE, Ringwood, Australia). Analysis conditions were: injector and detector temperature at 250°C, oven temperature at 160°C, and the carrier gas helium with a linear velocity of 49.7 cm/s (2.4 mL/min).

FAME were isolated by TLC on Kielselgel 60F 254, 20 × 20 cm, 0.2 mm thickness (Merck, Darmstadt, Germany), impregnated with silver nitrate (30%) as described by Aubert-Mammou *et al.* (12). The elution was performed by two successive developments in hexane/ether (80:20, vol/vol). The bands were visualized under ultraviolet radiation at 360 nm after spraying the plate with a 0.05% alcoholic solution of 2′,7′-dichlorofluorescein. The main bands were scraped off and extracted three times with boiling chloroform/methanol (90:10, vol/vol). The resulting solution was filtered and evaporated, and the purity of the four fractions obtained was checked by GLC. The third fraction, which yielded two components, was analyzed by HPLC (Kontron Instruments, Tegimenta, Switzerland). The chromatograph was fitted with a 25-cm RP 18 (octadecyl reversed-phase) column (Lichrosorb, Merck) with an internal diameter of 4 mm and a differential refractometer R401 (Waters Associates, Milford, MA). The eluent was an acetone/acetonitrile mixture (1:5, vol/vol) with a flow of 0.6 mL/min.

NMR spectra were obtained on a Jeol EX400 (Tokyo, Japan). Oils and FAME were studied in $CDCl_3$ in 5-mm NMR tubes. ^{1}H spectra, obtained at 399.65 MHz, with a sweep width of 8 KHz free induction decay (FID), were acquired at room temperature with a 45° excitation pulse and 65K data points. Tetramethylsilane (TMS) was used as internal standard.

^{13}C spectra were obtained at 100.4 MHz with 262K data points, a sweep width of 32 KHz and a 90° excitation pulse, with TMS or $CDCl_3$ (77.0 ppm) as reference. For 30 mg of oil, the ^{13}C spectrum was acquired at 35°C with an accumulation of 2000 scans and a pulse delay of 20 s. A gaussian

broadening filter of 0.25 Hz was applied to FID before Fourier transformation (FT). For FAME (5–15 mg), FID of ^{13}C spectra were obtained at room temperature with a pulse delay of 6 s and an acquisition time of 4.35 s. To obtain better sensitivity and resolution, an exponential broadening filter of 0.5 Hz was applied to the FID before FT.

Distortionless enhanced by polarization transfer (DEPT) spectra were obtained by using a 90° variable proton pulse, $\tau = 1/2J$ ($J \cong 140$ Hz) and 1.5 s relaxation delay. The FID was acquired with 32K data points, and an exponential filter of 1.0 Hz was applied before FT. For two-dimensional (2D) NMR, the experimental conditions were as follows: (i) H/H spectra: 256 experiments of 8 scans each, relaxation delay 1.0 s, acquisition time 0.16 s, $\Delta 1 = 0.3$ ms, $\Delta 2 = 1.0$ ms, spectral width in f2 and f1 3,000 Hz, exponential multiplication (broadening factor of 0.5 Hz); (ii) $^1H/^{13}C$ spectra: 128 experiments of 256 scans each, relaxation delay 1.0 s, acquisition time 0.25 s, $\Delta 1 = 3.6$ ms, $\Delta 2 = 1.8$ ms, spectral width in f2 16,300 Hz, in f1 3,500 Hz, exponential multiplication (broadening factor of 1.0 Hz); (iii) HMBC spectra: 256 experiments of 128 scans each, relaxation delay 1.4 s, acquisition time 0.34 s, $\Delta 1 = 3.57$ ms, $\Delta 2 = 62.5$ ms, spectral width in f2 18,500 Hz, in f1 3,000 Hz, square sine–bell multiplication.

RESULTS

Proton NMR studies of lipids or FAME have led to few publications (9) because there are not many characteristic signals. On the other hand, ^{13}C NMR has been developed considerably during recent years (13–15). This technique allows one to identify the nature of ethylenic fatty acids as well as the stereochemistry of double bonds. *Hydnocarpus* seed oil is characterized by the presence of cyclopentenyl fatty acids (90% of the mixture of the fatty acids). Identification of these fatty acids is difficult by GLC; their retention times could be confused with those of more commonly occurring fatty acids (stearic and linolenic acids). ^1H and ^{13}C spectra of the oil show some specific signals of these fatty acids.

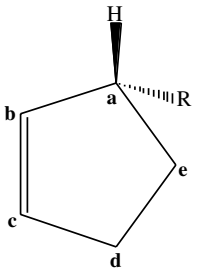
TABLE 1
Fatty Acid Composition (wt%) of *Hydnocarpus* Seed Oil

	Relative retention time			
Fatty acid	GLC[a]	HPLC[b]	R_f by Ag^+–TLC	(%)
16:0	1	1	0.69	3.3
16 cpe	1.67	1.26	0.47	47.3
18:1	1.79	1.65	0.57	1.1
18 cpe	2.91	1.84	0.47	35.4
18 cpde	3.17	1.18	0.17	10.5

[a]Retention time of palmitate methyl ester: 4.44 min.
[b]Retention time of palmitate methyl ester: 8.5 min. Abbreviations: cpe, cyclopent-2-enyl ring ; cpde, cyclopent-2-enyl ring and a double bond in the aliphatic chain; GLC, gas–liquid chromatography; HPLC, high-pressure liquid chromatography; Ag^+–TLC, silver-ion thin-layer chromatography.

To attribute the different peaks, we isolated the FAME by Ag^+–TLC and HPLC; these two techniques are complementary. By Ag^+–TLC, gorlic acid **3** was isolated with a purity of 100%. Hydnocarpic **1** and chaulmoogric **2** acids have the same R_f in Ag^+–TLC but different relative retention times (RRT) in HPLC and were purified by a combination of these two techniques (Table 1). Each FAME was studied by NMR, and different attributions were carried out by using appropriate spectroscopic techniques: 2D H/H, 2D H/C, DEPT, and HMBC. The FAME profile was determined by comparison between the different R_f and RRT in relation with the results obtained by NMR spectroscopy.

The DEPT technique allows one to distinguish secondary carbon atoms from other carbon atom types. Only one hybridized ternary sp^3 carbon atom is present among the compounds studied. This atom is observed at $\delta = 45.5$ ppm for the three cyclopentenyl fatty acids. Thus, it corresponds to the carbon atom **a** (Fig. 1); its chemical shift is not influenced by the nature of the chain (Table 2). According to the 2D H/C spectrum and different irradiation experiments, the signal at 45.5 ppm is correlated with the peak at 2.60 ppm, which can be assigned to the proton **Ha** without ambiguity. Other hydrogen atom chemical shifts were determined in the same way.



R = $-\overset{\alpha}{C}H_2-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_2-(CH_2)_7-\overset{3}{C}H_2-\overset{2}{C}H_2-\overset{1}{C}OOH$: Chaulmoogric acid

$-\overset{\alpha}{C}H_2-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_2-(CH_2)_5-\overset{3}{C}H_2-\overset{2}{C}H_2-\overset{1}{C}OOH$: Hydnocarpic acid

$-\overset{\alpha}{C}H_2-\overset{\beta}{C}H_2-\overset{\gamma}{C}H_2-(CH_2)_3-CH=\overset{6}{C}H-(CH_2)_2-\overset{3}{C}H_2-\overset{2}{C}H_2-\overset{1}{C}OOH$: Gorlic acid

FIG. 1. Fatty acid methyl esters of *Hydnocarpus* seed oil.

TABLE 2
Fatty Acid Methyl Esters of *Hydnocarpus* Seed Oil

Carbon number	^{13}C chemical shift (ppm)			^{1}H chemical shift (ppm) of cyclopentenyl fatty acids
	Chaulmoogric acid, C_{18} cpe	Hydnocarpic acid, C_{16} cpe	Gorlic acid, C_{18} cpde	
1	174.40	174.40	174.35	—
2	34.10	34.10	33.99	2.30
3	24.93	24.93	24.55	1.61
4	29.12	29.12	29.58	
5	29.43	29.43	26.78	
6	29.56	29.56	129.01	
7	29.62	29.62	130.44	
8	29.62	29.62	27.20	
9	29.84	—	29.18	
10	29.76	—	29.69	
γ	27.97[a]	27.97[a]	27.94[a]	
β	29.23[a]	29.23[a]	29.23[a]	
α	36.15	36.15	36.13	1.25/1.35[b]
a	45.58	45.58	45.56	2.60
b	135.45	135.43	135.43	5.68
c	129.97	129.94	129.98	5.68
d	31.96	31.94	31.96	2.30
e	29.87	29.85	29.84	2.02/1.27[b]

[a]These two signals could be interchanged.
[b]The protons of the carbon "α" and "e" resonate at different fields. For abbreviations, see Table 1.

By H/C correlation, we have attributed the cyclopentene ring carbon atoms. To differentiate **c** and **b** carbon atoms, we used the HMBC technique. By using J^2 H–C coupling constants, the HMBC method allowed us to correlate a hydrogen atom to a carbon atom separated by two bonds. The **Ha** proton is coupled with the signal at 135.4 ppm and thus corresponds to carbon atom **b**. Carbon atom **c** resonates at 129.94 ppm (Fig. 2).

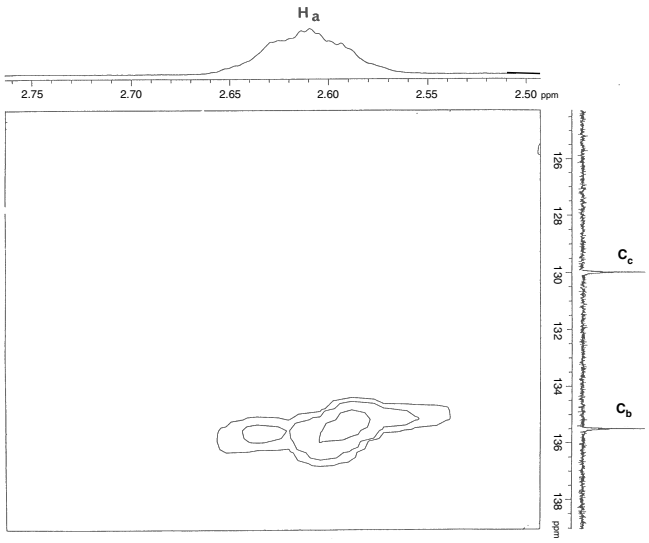


FIG. 2. ^{1}H/^{13}C long-range heteronuclear multiple bond correlation spectrum of the chaulmoogric acid. Expansion shows the ethylenic connectivities to proton $\mathbf{H_a}$.

These different spectral NMR studies allowed us to identify the hydrogen and carbon atom chemical shifts of the cyclopentene ring of gorlic, chaulmoogric, and hydnocarpic acids (Table 2). We noticed that these chemical shifts were not influenced by the nature of the fatty chain. However, those values are specific to the five elements of the ring and allowed us to show the presence of those acids in any oil.

NMR studies of the protons as well as of the carbons found other signals that correspond to methylene groups and/or the double bond of the fatty chain. Only the signal that corresponds to the methylenic group α to the ring is characteristic of the structure of those fatty acids and resonates in ^{1}H NMR at 1.25 and 1.35 ppm and in ^{13}C at 36.15 ppm. The other peaks observed in ^{1}H NMR (δ = 2.30 and 1.61 ppm) correspond to methylene groups in positions 2 and 3 on the chain. In the methyl gorlate spectrum, we observed also the signals due to the ethylenic double bond of the fatty chain. The HMBC study of this compound allowed the determination of the position of this double bond on carbon 6 of the fatty chain and confirmed the mass spectrometry analysis (8). We noticed that the chemical shifts obtained for the allylic and olefinic carbon atoms lead to the same conclusion as Gunstone's report (9). The other signals of compounds **1** and **2** resonate at fields similar to those observed for methyl stearate and palmitate. The chemical shift assignments are indicated in Table 2 according to the literature (16).

The ^{1}H and ^{13}C NMR study of hydnocarpic, chaulmoogric, and gorlic acid methyl esters, isolated from *Hydnocarpus* sp. seed oil, showed that these acids are easily identified in an oil by the presence of characteristic signals of the cyclopentenyl ring.

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