Separation and Identification of Ximenynic Acid Isomers in the Seed Oil of *Santalum spicatum* R.Br. as Their 4,4-Dimethyloxazoline Derivatives

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ABSTRACT: The seed oil of *Santalum spicatum* contains a significant amount of ximenynic acid, *trans*-11-octadecen-9-ynoic acid, a long-chain acetylenic fatty acid, as a major component (34%). The identity of *trans*-ximenynic acid was confirmed after isolation by ultraviolet, infrared, and nuclear magnetic resonance (NMR) (^{1}H- and ^{13}C-) spectroscopy and by gas chromatography/mass spectrometry (GC/MS). The *cis* isomer of ximenynic acid was also found (<1%) in some samples. The *cis* and *trans* isomers were characterized by GC/MS comparison of their methyl esters and 4,4-dimethyloxazoline derivatives. *JAOCS 73*, 1729–1731 (1996).

KEY WORDS: 4,4-Dimethyloxazoline, fatty acid methyl ester, gas chromatography, isomers, sandalwood (*Santalum spicatum*), ximenynic acid.

The fatty acid (FA) composition of the seed oil of the West Australian sandalwood (*Santalum spicatum* R.Br.) was analyzed by gas chromatography/mass spectrometry (GC/MS) of their methyl esters. In addition to the common C_{16} and C_{18} fatty acids, such as palmitic and oleic acids, a distinctive feature is the presence of the acetylenic fatty acids ximenynic acid (*trans*-11-octadecen-9-ynoic acid) and stearolic acid (9-octadecynoic acid) (1). During the FA methyl esters (FAME) analysis of the seed oil, some samples displayed an unknown peak that had the same mass spectrum as *trans*-ximenynic methyl ester. It was presumed that the peak represented the less common *cis*-isomer of ximenynic acid, which has been detected in other plant species, such as Isano, *Ongokea gove* (2), and *Curupira tefeensis* (3). The identify of this compound was confirmed with a derivatization method by reaction with 2-amino-2-methyl-1-propanol (AMP) to form the 2-substituted 4,4-dimethyloxazoline (DMOX) compound (4–6). Use of the DMOX derivatives of FA enables a precise location of double and triple bonds to be made (5).

This paper describes the separation and identification of *cis* and *trans* isomers of ximenynic acid in the seed oil of *S. spicatum*.

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EXPERIMENTAL PROCEDURES

Oil extraction. Mature seeds of *S. spicatum* were harvested in late October 1993 from 12-year-old trees, grown under irrigation conditions in the Field-Trial Area, School of Environmental Biology, Curtin University of Technology (Perth, Australia). The seeds were maintained at about 20°C before analysis.

Seed samples of approximately 2 g were ground with a pestle and mortar and extracted with 200 mL hexane in a Soxhlet apparatus (Quickfit^R, Stone, Staffordshire, England) for 2 h. The solution was dried with anhydrous sodium sulfate, and the solvent was removed in a Buchi rotatory evaporator (Flawil, Switzerland) under reduced pressure at 60°C to yield 55.31% of a viscous, pale yellow oil.

Isolation of trans-ximenynic acid. Following the hydrolysis method described by Christie (7), a sample of the oil (10 g) was refluxed with 1 M solution of potassium hydroxide in 95% ethanol (200 mL) for 1 h. The mixture was cooled and acidified with 6 M HCl to pH 1. The free FA were extracted with hexane, and the solution was dried over anhydrous sodium sulfate and evaporated in a Buchi rotatory evaporator under reduced pressure at 60°C to yield the free FA as a viscous oil (8.64 g). Ximenynic acid was obtained by crystallization from hexane at −25°C (8). Recrystallization from hexane gave *trans*-ximenynic acid as white flakes, melting point 38.9–39.3°C [38.5–39.5°C (9)].

FA derivatives. Identity of the FA in the oil was determined by preparation and analysis of FAME and DMOX derivatives. The FAME were prepared in a mixture of toluene (2 mL) and 1% sulfuric acid in methanol (4 mL) at 50°C overnight (7). The DMOX derivatives of the total FA (obtained by alkaline hydrolysis of the oil) were prepared by heating 0.1 mL of FA solution (1 mg FA dissolved in 1 mL toluene) with 0.05 mL of AMP for 2 h at 210°C in a muffle furnace in a sealed, capped tube (4,6).

FA analysis. FA derivatives (FAME and DMOX) were quantitated by GC/MS in a Hewlett-Packard (Palo Alto, CA) 5890 Series 11 GC with an HP 5971 mass-selective detector. The separation was carried out on a DB 23 (J&W Scientific, Folsom, CA) capillary column (30 m × 0.25 mm i.d., film thickness 0.15 μm) at 50°C for 5 min, then 5°C per min to

240°C, which was maintained for a further 5 min. Helium was used as carrier gas with the linear velocity controlled to 47.5 cm s^{-1} (1.40 mL min^{-1}) by an electronic pressure program with vacuum compensation.

The ultraviolet (UV) spectrum of *trans*-ximenynic acid in hexane was obtained with an HP Model 8452A PC UV/visible scanning spectrophotometer. Infrared (IR) spectra were recorded on a Bruker (Karlsruhe, Germany) IFS 66 spectrophotometer with *trans*-ximenynic acid methyl ester as a thin film and the free acid as a melted solid between NaCl plates. ^{1}H- and ^{13}C-NMR spectra of *trans*-ximenynic acid methyl ester in C_6D_6 solution were obtained with a Bruker (Rheinstetten, Germany) AN300 NMR spectrometer. The ^{13}C signals were obtained as composite pulse-decoupled spectra.

RESULTS AND DISCUSSION

GC/MS analysis of FAME showed the presence of two ximenynic acid peaks (M^+ *m/z* 292, consistent with the molecular formula $C_{19}H_{32}O_2$) at retention time (RT) 36.65 min. (34%) and RT 34.60 min. (0.3%). The mass spectra of both peaks were concordant with our normal expectation of a ximenynic acid (11-octadecen-9-ynoic acid) methyl ester. Significant features of the mass spectra included fragments at *m/z* 261 [$(M-31)^+$, loss of CH_3O], *m/z* 219 [$(M-73)^+$, loss of $CH_2CO_2CH_3$], and novel fragments at *m/z* 164 [$H-(CH=CH)_2-CH=CH(CH_2)_5CH_3]^+$ and *m/z* 150 [$CH_2=C=CH-CH=CH(CH_2)_5CH_3]^+$, both indicative of a C_{18}-enyne structure with the triple bond between C_9 and C_{10} (3,10). The most intense ion was at *m/z* 79 $(C_6H_7)^+$ and indicated the presence of more than one double or triple bond in the long-chain structure (11). It was felt that unequivocal identification of these compounds was not possible solely in terms of mass spectral fragmentation of the FAME derivatives, and recourse was thus made to other spectral evidence and the use of DMOX derivatives.

That most of the ximenynic acid was of the *trans* configuration was confirmed by first isolating a sample of ximenynic acid by the hydrolysis procedure of Christie (7), including steps of alkaline hydrolysis, then acidification, solvent extraction, and multiple recrystallization from hexane to constant melting point. The UV spectral analysis of *trans*-ximenynic acid in hexane showed a λ_{max} at 229 nm, which is indicative of a conjugated system (9,12). The IR absorption at 955 cm^{-1} of ximenynic methyl ester and 956 cm^{-1} of the free acid provided confirmation of the *trans* double bond present in a conjugated system (12,13). The ^{1}H-NMR spectrum of the ximenynic acid methyl ester in C_6D_6 showed a high degree of correspondence to that previously reported (12). In particular, the spectrum showed a complex doublet centered at 5.56 ppm, *J* 15.7 Hz and a doublet-triplet centered at 6.14 ppm, J_1 15.7 Hz, J_2 7.1 Hz, which can be assigned to the *trans*-alkenic protons of a conjugated enyne system (12,14). The ^{13}C-NMR spectrum also showed a high degree of correspondence to details previously reported (12) and exhibited singlets at 80.06 and 88.89 ppm, which were assigned to the acetylenic carbon atoms, C_9 and C_{10}, and at 110.91 and 143.11 ppm, which were assigned to the olefinic carbon atoms, C_{11} and C_{12} (12,15).

The minor peak at RT 34.60 min gave a mass spectrum similar to that of the *trans*-ximenynic acid methyl ester, confirming its general identity as a ximenynic acid. The similarity of the FAME mass spectra, while indicative of the relationship between the two compounds, could not be presumed to be absolutely specific to a ximenynic acid identity due to the possibilities for bond rearrangement of FAME derivatives (7). Recourse was thus made to the use of the stable DMOX derivatives.

The GC of the fatty acid DMOX mixture also showed an additional minor peak at RT 36.33 min with a mass spectrum that was characteristic of the major ximenynic acid DMOX derivative peak at RT 38.51 min. The mass spectrum of the minor ximenynic acid DMOX derivative (RT 36.33 min) is shown in Figure 1 and compares favorably with that of the major *trans*-ximenynic acid DMOX derivative shown in Figure 2. Examination of the mass spectrum of the minor ximenynic acid DMOX derivative shows a molecular ion at *m/z* 331 (calculated $C_{22}H_{37}NO$), and characteristic fragments at *m/z* 113 [resulting from a McLafferty rearrangement (4)], 126, 140, 182, and 274. The fragment at *m/z* 274 is specific to ximenynic acid DMOX in sandalwood seed oil, produced by fission at C_{14}–C_{15}. Certain fragments were not seen due to the low abundance of this minor peak. The spectrum of the more abundant *trans*-ximenynic acid also shows a characteristic set of homologous ions at *m/z* 126 + $(14)_n$, together with mass differences of 12 atomic mass units (amu) between the low-intensity fragments at *m/z* 232 (C_{11}) and 220 (C_{10}) and 10 amu between those at *m/z* 206 (C_9) and 196 (C_8) which indicate conjugated triple and double bonds at carbons 9 and 11, respectively (5).

According to previously published data (3,5), the mass spectrum observed for the DMOX minor peak at RT 36.33 min confirms our conclusion that the unknown peak is the *cis*-isomer of ximenynic acid (*cis*-11-octadecen-9-ynoic acid). Further data analysis of the FA DMOX derivatives chromatogram with a selected ion chromatogram at *m/z* 274 con-

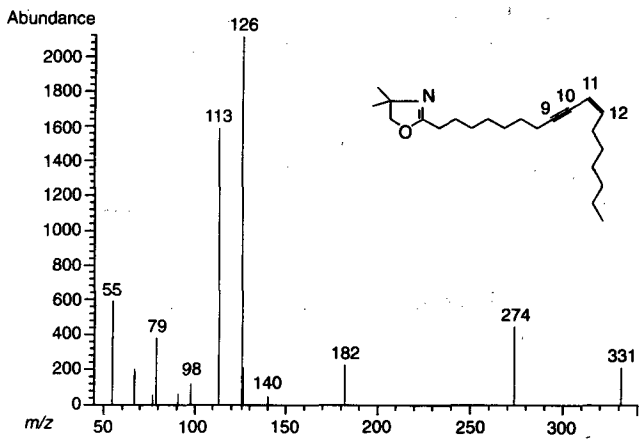

FIG. 1. Mass spectrum of the DMOX derivative of *cis*-11-octadecene-9-ynoic acid.

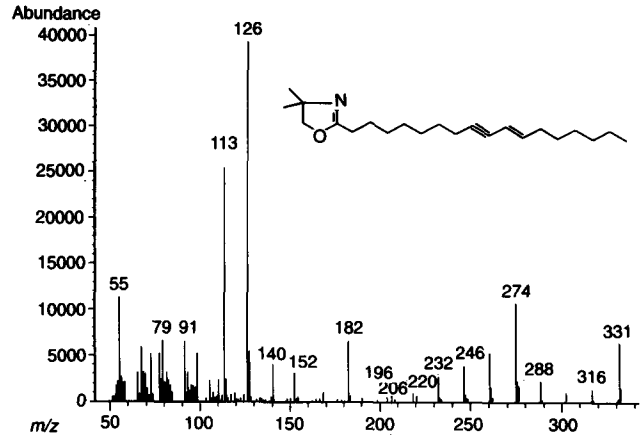

FIG. 2. Mass spectrum of the DMOX derivative of *trans*-11-octadecene-9-ynoic acid.

firmed the presence of only the two geometrical isomers of ximenynic acid as discussed, and the absence of any other FA with that characteristic fragment. The order of retention of the *cis*- and *trans*-ximenynic methyl esters was in good agreement with previous findings reported for *Curipira tefeenis* seed oil (3), which were carried out under similar conditions to those described in this study, and where the DMOX derivative of the *cis*-ximenynic acid eluted before the DMOX derivative of *trans*-ximenynic acid, by several minutes.

The natural co-occurrence of geometrical isomers of FA in plant-fixed oils as a product of specific metabolic processes is not uncommon, being particularly exemplified by oleic (*cis* $C_{18:1n9}$) and elaidic (*trans* $C_{18:1n9}$) acids. Our analysis of sandalwood seed oil routinely demonstrates that *cis*-ximenynic acid appears to be a normal component, even though occurring at low concentration (about 0.3%). The detection and characterization of *cis*- and *trans*-ximenynic acids in *C. tefeensis* (3) has substantiated the fact that the *cis* isomer may also occur naturally in other plants.

ACKNOWLEDGMENTS

Yandi Liu has received a Curtin University Overseas Postgraduate Research Scholarship (1993–1996). The technical support of Bruce MacKinnon and Peter Chapman is appreciated. Dr. Lindsay Byrne, Department of Chemistry, University of Western Australia, performed the NMR spectral determinations. Dr. Volker Spitzer, Porto Alegro, Brazil, provided valuable data and chromatography information on *cis*-ximenynic acid in *Curupira tefeensis*.

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[Received February 26, 1996; accepted September 11, 1996]