Cyclopropenoic Fatty Acids in Gymnosperms: The Seed Oil of *Welwitschia*

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ABSTRACT: The seed oil of the gymnosperm *Welwitschia mirabilis* was found to contain malvalic acid, a cyclopropenoic fatty acid. This is in sharp contrast to most other gymnosperms, which contain Δ5*cis*-fatty acids as well as the normal set of fatty acids. The importance of this finding in relation to questions of the evolution of the Gymnospermae and Angiospermae, the two main branches of higher plants, is briefly discussed.
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KEY WORDS: Angiosperms, *Cimicifuga*, cyclopropenoic fatty acids, *Ephedra*, evolution, *Gnetum*, gymnosperms, malvalic acid, *Pseudotsuga*, *Welwitschia*.

The finding of malvalic acid (18:1Δ8cpe, where cpe = cyclopropenoic) in the seed oil of southeast-Asian *Gnetum scandens* by Berry (1) came as a surprise to most researchers familiar with the fatty acid composition of seed oils of Gymnospermae. Originally, this cyclopropenoic acid was identified by the use of Halphen and HBr reactions, but more recently, Vickery (2) and Mustafa *et al.* (3) confirmed the occurrence of malvalic acid in *Gnetum* by gas chromatographic techniques.

Cyclopropenoic fatty acids are known to occur primarily in representatives of the angiosperm plant order Malvales, but *Gnetum* is a member of one branch of the much older gymnosperms. Gymnosperms, however, are known to contain Δ5-fatty acids—such as 20:3Δ5*cis*,11*cis*,14*cis* and pinolenic acid (18:3Δ5*cis*,9*cis*,12*cis*)—as the only unusual types of fatty acids, as well as the normal set of fatty acids (4–7). Whereas pinolenic acid seems to be limited to the conifers, and in particular to the Pinaceae, 20:3Δ5*cis*,11*cis*,14*cis* appears to be a nearly universal constituent of gymnosperm seed oils and also of even more archaic plants, such as *Equisetum* (4). This fatty acid was also found in three species of *Ephedra*, and in all other gymnosperm genera investigated so far except *Gnetum* (1–7). More recent work on other *Ephedra* spp. (Wolff, R.L. *et al.*, unpublished data) supported its presence as a major constituent of *Ephedra* seed oils. However, the levels of this fatty acid were low in the seed oils of a few *Pinus* species (7). *Gnetum, Ephedra,* and *Welwitschia* are all members of the same branch of the Gymnospermae (the order Gnetales), and they are believed to be the closest living relatives of the Angiospermae (8–10).

For *Welwitschia,* the occurrence of the normal fatty acids only had been reported by Daulatabad *et al.* (11). *Welwitschia mirabilis* Hook.f. is a rare and strange species (a monotypic family and genus), which occurs only in the desert regions of Namibia and Angola.

In this paper, new findings are reported on *Welwitschia,* as well as on *Ephedra* and some other gymnosperm seed oils. The results are discussed briefly in light of present evidence, both on gymnosperm seed oil fatty acid composition and on Gymnospermae and Angiospermae evolution.

MATERIALS AND METHODS

Seed materials. Seeds of *W. mirabilis* were kindly supplied by the Botanical Garden of the University of Stellenbosch, South Africa. Seeds of *Ephedra gerardiana* were collected in the Botanical Garden of the University of Hamburg. Seeds of *Pseudotsuga menziesii* (Douglas fir) were collected at full ripeness from plants in a local garden. Seeds of *Cimicifuga* spp. were from our earlier investigation of the Ranunculaceae (12,13).

Gas–liquid chromatography (GLC) of fatty acid methyl esters. The extraction of seed lipids, formation of fatty acid methyl esters, and GLC on a Silar 5CP column were carried out as described for chemotaxonomically relevant standardized "seed oil fatty acid fingerprints" (12,14) and for our screening program for γ-linolenic acid in seeds of wild plants (15–21). However, because only a small amount of seeds was available and because the fat content was low, the unsaponifiable matter was not separated prior to BF_3/methanol esterification. The quantitative analysis of cyclopropenes was carried out after $AgNO_3$/methanol rearrangement derivatization as described (22,23).

Gas chromatography–mass spectrometry (GC–MS) of malvalic acid derivative. GC–MS analysis of the methyl ether rearrangement products (24,25) was carried out by the electron ionization mode (EI; 70 eV) on a Hewlett-Packard instrument Model 5890 Series II/5989 A (Palo Alto, CA), equipped with a 0.23 µm Permabond OV-1 fused-silica capillary column (Macherey-Nagel, Duren, Germany), 25 m × 0.32 mm i.d. The carrier gas was helium at a flow rate of 1.0 mL/min. The column temperature was initially kept at 140°C

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TABLE 1
Fatty Acid Composition of *Welwitschia mirabilis* Seed Oil Before and After $AgNO_3$/Methanol Derivatization, of *Ephedra gerardiana* Seed and Pulp Lipids, and of *Pseudotsuga menziesii* and *Cimicifuga racemosa* Seed Oils

	Welwitschia mirabilis		*Ephedra gerardiana*		*Pseudotsuga menziesii*	*Cimicifuga racemosa*
	seed oil[a]	seed oil[b]	seed lipids	pulp lipids	seed oil	seed oil
Lipid (wt%)	18.2		15.7		18.8	35.0
Fatty acids						
10:0	—	—	0.1	1.1	trace	—
12:0	—	—	trace	1.2	—	trace
13:0	—	—	—	0.1	—	—
14:0	0.2	0.2	0.1	3.0	0.1	0.1
14:1n-5	—	—	0.1	—	trace	—
15:0	0.1	0.1	0.1	0.3	trace	trace
16:0	21.4	21.3	6.6	21.1	3.5	5.4
16:1Δ5*c*	—	—	—	—	—	1.1
16:1n-9	0.1	0.1	0.1	0.3	0.1	—
16:1n-7	0.7	0.7	0.6	0.8	0.2	trace
17:0 anteiso	—	—	—	—	1.3	—
17:0	0.6	0.6	0.1	0.1	0.1	trace
17:1n-7	—	—	trace	—	trace	—
9,10-methylene-16:0	1.6	1.6	—	—	—	—
18:0	2.7	2.7	2.5	3.4	1.8	2.6
18:1Δ5*c*	—	—	—	—	—	1.9
Malvalic	6.0	—	—	—	—	—
18:1n-9	11.1	11.0	17.1	16.0	18.1	7.0
18:1n-7	1.8	1.8	11.2	1.4	0.8	0.2
18:2Δ5*c*,9*c*	0.3	0.3	0.4	0.2	2.8	—
18:2Δ5*c*,11*c*	—	—	1.7	0.1	—	—
18:2n-6	15.1	15.0	8.7	24.0	44.0	29.0
18:2n-4/19:0	trace	trace	0.1	0.2	—	0.1
18:3Δ5*c*,9*c*,12*c*	—	—	—	—	20.1	—
Sterculic	0.6	—	—	—	—	—
9,10-methylene-18:0	0.2	0.2	—	—	—	—
18:3n-3	33.5	33.6	10.5	18.8	0.6	8.2
18:4Δ5*c*,9*c*,12*c*,15*c*	0.1	0.1	0.5	—	0.1	0.9
20:0	0.2	0.2	0.4	0.4	0.6	1.8
20:1Δ5*c*	—	—	—	—	—	0.4
20:1n-11	—	—	—	0.1	—	—
20:1n-9	0.1	0.1	0.5	0.3	0.9	18.6
20:1n-7	—	—	0.5	0.1	—	—
20:2Δ5*c*,11*c*	—	—	1.5	0.1	0.4	0.8
20:2n-6	—	—	2.6	0.2	0.5	4.8
Rearranged malvalic	—	5.6	—	—	—	—
20:3Δ5*c*,11*c*,14*c*	—	—	7.5	1.4	1.7	5.8
20:3n-3	—	—	3.3	trace	—	2.2
20:4Δ5*c*,11*c*,14*c*,17*c*	—	—	19.2	0.3	—	8.0
Rearranged sterculic	—	0.6	—	—	—	—
22:0	0.2	—	0.2	0.3	0.5	0.3
22:1n-11	—	—	—	0.5	—	—
22:1n-9	—	—	trace	—	0.1	trace
x (ketone ?)	—	0.5	—	—	—	—
23:0	—	—	—	0.1	0.1	—
24:0	0.3	0.3	0.1	0.4	0.2	0.1
26:0	—	—	0.1	0.3	0.1	—

[a] Esterified with BF_3/CH_3OH.
[b] Esterified with BF_3/CH_3OH, followed by $AgNO_3/CH_3OH$ rearrangement derivatization.

for 5 min, then programmed from 140 to 260°C at 4°/min. The final temperature was held for 5 min. Other operating conditions were a split/splitless injector (split 1:20, temperature 280°C), an interface temperature of 280°C, and an ion source temperature of 200°C.

RESULTS

The fatty acid compositions of the seed oils from the species investigated here are shown in Table 1. Malvalic and sterculic acids were found at levels of 6.0 and 0.6%, respectively, be-

fore $AgNO_3$ rearrangement and at 5.6 and 0.6% after rearrangement (Table 1). They were present only in *Welwitschia.* Sterculic acid is known to coincide with γ-linolenic acid (18:3n-6) in our standardized GLC "fingerprint" system with the Silar 5CP column (14). Likewise, peak *x* (in Fig. 1 and Table 1) is most likely one of the ketone derivatives of malvalic acid rearrangement (22–25), although it coincides with the retention time of 22:2n-6. This peak had the same GLC retention time as the known major ketone derivative obtained from malvalic acid in *Hibiscus* seed oil fatty acid methyl esters, when these were treated with $AgNO_3$/acetonitrile in the absence of methanol. Figure 1 shows a superposition of the

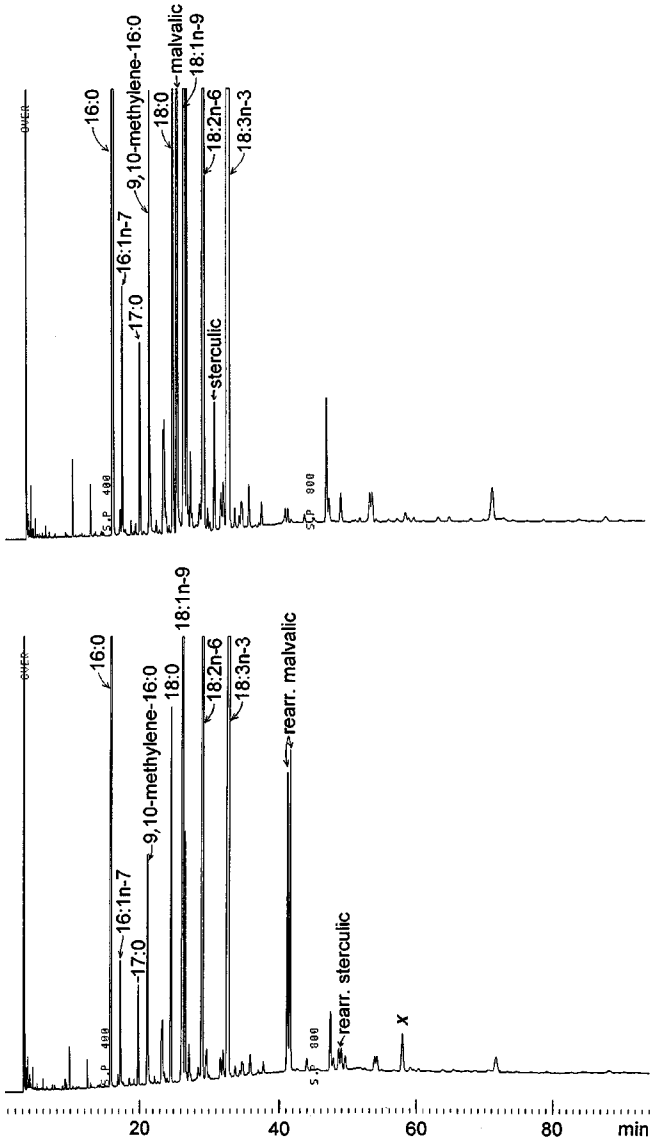

FIG. 1. Gas–liquid chromatographic fatty acid methyl ester fingerprints (14) of *Welwitschia mirabilis* seed oil before and after $AgNO_3$/methanol rearrangement (22,23). A Silar 5 CP column and the standardized temperature gradient for seed oil "fatty acid fingerprints" was used as described (12,14). Peak *x* is most likely a ketone derivative of malvalic acid.

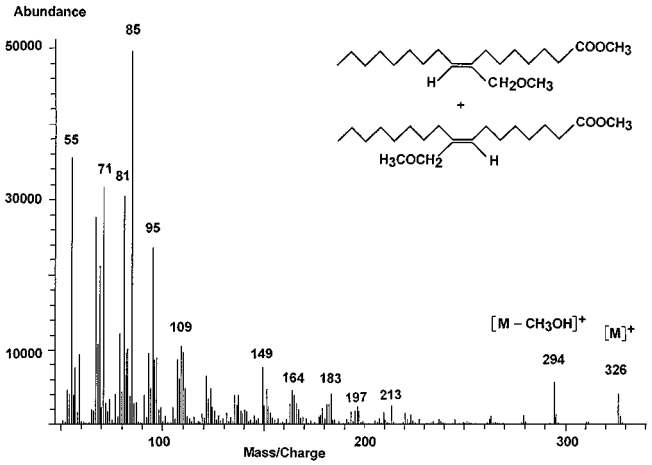

FIG. 2. Mass spectrum and structure of the $AgNO_3$/methanol methyl ether rearrangement products obtained from *W. mirabilis* fatty acid methyl esters. The fragmentation corresponds to that observed by Eisele *et al.* [cf. Fig. 8 in their publication (25)] and Ahmad *et al.* (24).

seed oil fatty acid fingerprints (14) obtained from *W. mirabilis* directly before and after $AgNO_3$/methanol rearrangement (22,23). For comparison, Table 1 also shows the seed oil fatty acid composition obtained from *E. gerardiana* (Ephedraceae, another gymnosperm, also belonging to the order Gnetales) and *C. racemosa* (Ranunculaceae, an angiosperm). The GC–MS fragmentation of the $AgNO_3$/methanol derivatization product of malvalic acid as obtained from *Welwitschia* is illustrated in Figure 2.

The presence of malvalic acid was established by GLC of the fatty acid methyl esters before and after $AgNO_3$/methanol rearrangement and by direct comparison with the cyclopropenoic fatty acids of *Hibiscus syriacus* and *Althaea officinalis* (Malvaceae), including their rearrangement products. Both the GLC behavior of intact malvalic acid methyl ester and the formation (and GLC) of the various degradation and rearrangement products obtained from malvalic and other cyclopropenoic fatty acid methyl esters are well known and have been described repeatedly in the relevant literature (22–37).

The structures of malvalic and sterculic acid were confirmed by GC–MS of their ether rearrangement products. The mass spectra of the ether rearrangement products of malvalic acid are well known (24,25). They have base peaks of *m/z* 85 and intense peaks at *m/z* 55, 71, 81, and 95. Besides the molecular ion at *m/z* 326 (which in itself confirms the addition of methanol to a C_{18} cyclopropenoic fatty acid methyl ester), there is also a characteristic fragment of parent minus 32 mass units present in the spectrum, which indicates the loss of methanol from the molecular ion. One of the expected ketone derivatives, which are often produced at low levels only (23–28), may be present as peak *x* in Figure 1 and Table 1, but this could not be confirmed by GC–MS because significant diagnostic peaks were known to be absent from their spectra.

DISCUSSION

Much has been published on cyclopropenoic fatty acid contents of seeds and seed oils. Unfortunately, both false negatives and false positives seem to abound, particularly in the older literature. Analysis of cyclopropenoic fatty acids is difficult because these compounds are rather labile (23,25,29–32). The presence of these acids may simply have been overlooked during the analysis of *Welwitschia* carried out by Daulatabad *et al.* (11), or they may have been destroyed during sample preparation and thus were not identified. On the other hand, it seems that these authors had not even used GLC (at least they do not mention it), and it is unclear how the results presented in their paper (11) were obtained. It is well known that cyclopropenoic fatty acids require special precautions for their analysis (22,29,30,33–37). If one is unaware of the presence of cyclopropenes or their decomposition products, the fatty acid methyl ester gas chromatograms obtained from such unknown oils can be rather difficult to interpret. In an earlier study (Aitzetmüller, K., unpublished data), the many side reactions during methyl ester formation from cyclopropenoic fatty acids, using different methylation techniques, were investigated with seed oils from *A. officinalis*, *Alcea rosea*, and *H. syriacus* (Malvaceae)—oils that are known to contain high levels of cyclopropenes.

Ephedra (Ephedraceae), *Gnetum* (Gnetaceae), and *Welwitschia* (Welwitschiaceae) together form the order Gnetales of the Gymnospermae, which seems to have evolved separately for the last 300 million yr. It is assumed that the Angiospermae may have evolved from a common ancestor to this branch of the Gymnospermae, living about 100–150 million yr ago (38), and that the Gnetales are now the "closest living relatives" of the angiosperms (8–10). In this context, the monophyletic or polyphyletic origin of the angiosperms has also been discussed (39,40).

Seed oils of *Ephedra* were investigated early and showed a fatty acid composition similar to that of many gymnosperms. The presence of C_{20}-fatty acids with 3–5 double bonds was established by Litchfield and others (41,42) in *E. nevadensis* and *E. campylopoda*. By using a sample from Mongolia, their findings were essentially confirmed when the seed oil "fatty acid fingerprint" of *E. przewalskii,* a gymnosperm, was investigated and compared with that of *Caltha palustris* (Ranunculaceae), an angiosperm (4). It was pointed out that similar fatty acid patterns were obtained from some gymnosperms (including *Ephedra*) and from some archaic members of the Ranunculaceae. The conspicuous absence of pinolenic acid (18:3Δ5*cis*,9*cis*,12*cis*) in these fatty acid patterns was considered another highly significant feature of both fingerprints (4). Δ5-Non-methylene-interrupted polyenoic (NMIP)-fatty acids with 20 carbon atoms, such as 20:3 and 20:4 with a Δ5 double bond, were often found in gymnosperms and in the more archaic members of the Ranunculaceae, such as *Caltha*, *Cimicifuga*, *Actaea*, *Eranthis*, and others (4,12,13,43,44). In four or five cases, there is evidence that both features, Δ5 desaturation and C_{20} chain elongation, may have gradually been lost during further steps of evolution of the Ranunculaceae (Aitzetmüller, K., unpublished data).

For *Welwitschia,* Daulatabad *et al.* (11) had claimed the presence of normal fatty acids only. However, much to our surprise, the reinvestigation of *Welwitschia* seed oil, reported here, unexpectedly established the presence of malvalic acid (18:1Δ8cpe) in the seed oil of this unusual gymnosperm. This finding means that both the Welwitschiaceae and Gnetaceae seed oils may contain cyclopropenoic fatty acids, as do the seed oils of most members of the order Malvales in the angiosperms. The Ephedraceae seed oils, on the other hand, are obviously more similar to those from the mainstream of the Gymnospermae; at the same time, they have much in common with a number of seed oils from the angiosperm plant family Ranunculaceae. It has also been noted that, in a number of other morphological and phytochemical aspects, *Welwitschia* and *Gnetum* appear to be related to each other, more closely than to *Ephedra* (45) and other gymnosperms. The present finding of malvalic acid in both *Welwitschia* and *Gnetum*, but not in *Ephedra* and in other Gymnospermae, is additional evidence for a closer relation of the former two genera.

A parallel evolution of Δ5-NMIP fatty acids (first in the Gymnospermae—including *Ephedra*—and later independently in the Ranunculaceae) is possible, of course. A parallel evolution of cyclopropenes, combined with the loss of Δ5-desaturation, both in *Gnetum* and *Welwitschia* and, separately and independently, in the Malvales is also a possibility—although it may seem less likely the more such analyses and coincidences become known. The early evolution of angiosperms may yet turn out to be more complicated than was thought previously, and perhaps even the question of Angiospermae monophyly (and that of Gnetales monophyly) should be discussed again.

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