Δ5-Olefinic Acids in the Seed Lipids from Four *Ephedra* Species and Their Distribution Between the α and β Positions of Triacylglycerols. Characteristics Common to Coniferophytes and Cycadophytes

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ABSTRACT: The fatty acid compositions of the seed lipids from four *Ephedra* species, *E. nevadensis, E. viridis, E. przewalskii,* and *E. gerardiana* (four gymnosperm species belonging to the Cycadophytes), have been established with an emphasis on Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA). Mass spectrometry of the picolinyl ester derivatives allowed characterization of 5,9- and 5,11-18:2; 5,9,12-18:3; 5,9,12,15-18:4; 5,11-20:2; 5,11,14-20:3; and 5,11,14,17-20:4 acids. Δ5-UPIFA with a Δ11-ethylenic bond (mostly C_{20} acids) were in higher proportions than Δ5-UPIFA with a Δ9 double bond (exclusively C_{18} acids) in all species. The total Δ5-UPIFA content was 17–31% of the total fatty acids, with 5,11,14-20:3 and 5,11,14,17-20:4 acids being the principal Δ5-UPIFA isomers. The relatively high level of *cis*-vaccenic (11-18:1) acid found in *Ephedra* spp. seeds, the presence of its Δ5-desaturation product, 5,11-18:2 acid (proposed trivial name: ephedrenic acid), and of its elongation product, 13-20:1 acid, were previously shown to occur in a single other species, *Ginkgo biloba,* among the approximately 170 gymnosperm species analyzed so far. Consequently, Ephedraceae and Coniferophytes (including Ginkgoatae), which have evolved separately since the Devonian period (~300 million yr ago), have kept in common the ability to synthesize C_{18} and C_{20} Δ5-UPIFA. We postulate the existence of two Δ5-desaturases in gymnosperm seeds, one possibly specific for unsaturated acids with a Δ9-ethylenic bond, and the other possibly specific for unsaturated acids with a Δ11-ethylenic bond. Alternatively, the Δ5-desaturases might be specific for the chain length with C_{18} unsaturated acids on the one hand and C_{20} unsaturated acids on the other hand. The resulting hypothetical pathways for the biosynthesis of Δ5-UPIFA in gymnosperm seeds are only distinguished by the position of 11-18:1 acid. Moreover, ^{13}C nuclear magnetic resonance spectroscopy of the seed oil from two *Ephedra* species has shown that Δ5-UPIFA are essentially excluded from the internal position of triacylglycerols, a characteristic common to all of the Coniferophytes analyzed so far (more than 30 species), with the possibility of an exclusive esterification at the *sn*-3 position. This structural feature would also date back to the Devonian period, but might have been lost in those rare angiosperm species containing Δ5-UPIFA.

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Gymnosperms are generally divided into two main taxonomic groups: the Coniferophytes and the Cycadophytes (1,2). Despite the fact that not all authors agree on this classification, what is important is that extant species belonging to these two distinct groups have evolved independently since the Devonian period for approximately 300 million years (1,2). The monophyletic taxon status of gymnosperms is doubtful (1). Takagi and Itabashi (3) have analyzed in detail the seed lipids from 21 gymnosperm species, including two Cycadophytes, whereas Wolff and coworkers have established the fatty acid composition of seed lipids from approximately 170 Coniferophyte species of the families Pinaceae, Cupressaceae, Taxodiaceae, Sciadopityaceae, Taxaceae, Cephalotaxaceae, Podocarpaceae (4–14; Wolff, R.L., Pédrono, F., and Marpeau, A.M., unpublished results), and more recently, of the family Araucariaceae (Wolff, R.L., Christie, W.W., Pédrono, F., and Marpeau, A.M., unpublished results).

Both Japanese and European researchers agreed on the systematic presence of Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA or Δ5-olefinic acids) in all analyzed gymnosperm seeds. The same peculiarity would also hold true for gymnosperm leaves (15). The following structures of Δ5-UPIFA have been characterized in gymnosperm plants: 5,9-18:2 (taxoleic); 5,11-18:2; 5,9,12-18:3 (pinolenic); 5,9,12,15-18:4 (coniferonic); 5,11-20:1; 5,11,14-20:3 (sciadonic), and 5,11,14,17-20:4 (juniperonic) acids, all ethylenic bonds being in the *cis* configuration. The content and profile of individual Δ5-UPIFA depend on the family, the genus, or even the species considered (5–8). The same Δ5-UPIFA (and others, e.g., 5,13-22:2 acid) have been observed in the seeds of about a dozen angiosperm species, which may additionally

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Abbreviations: Ag-TLC, argentation thin-layer chromatography; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TAG, triacylglycerol; UPIFA, unsaturated polymethylene-interrupted fatty acid.

contain Δ5-monoenoic acids, and *trans* Δ5-UPIFA are known in two or three angiosperm species (16–26).

The first report on the fatty acid composition of the seed lipids from a Cycadophyte species was probably that of Kleiman *et al*. (27), which was published more than 30 yr ago. The species under analysis was *Ephedra campylopoda*, and it was shown that the seeds contained several Δ5-UPIFA, i.e., 5,11-18:2; 5,11-20:2; 5,11,14-20:3, and 5,11,14,17-20:4 acids. The position of the ethylenic bonds as established by a complex combination of preparative or analytical gas–liquid chromatography (GLC) of the fatty acid methyl esters (FAME), fractionation according to the number of ethylenic bonds by argentation thin-layer chromatography (Ag-TLC), partial hydrazine reduction, and ozonolysis. The double bond location in eicosatetraenoic acid was later confirmed by GLC–mass spectrometry (MS) of its monomethoxy eicosanoates (28). With few discrepancies, the Δ5-olefinic acids identified in *E. campylopoda* seed lipids were the same as those characterized a few years earlier by Schlenk and Gellerman (29) in *Ginkgo biloba* seeds and leaves. Litchfield (30) analyzed the seed lipids from another Ephedraceae, *E. nevadensis* (known as Nevada Mormon tea), at approximately the same time, and though the exact location of the ethylenic bonds in each acid was not determined, he concluded that *E. nevadensis* seed fatty acids apparently resembled those identified in *E. campylopoda* by Kleiman *et al*. (27).

In the 1980s, some authors published supplementary data for the seeds and/or leaves of a few other Cycadophytes, i.e., *Welwitschia mirabilis* (31), *Gnetum scandens* (32), *Gnetum gnemon* (33), and *Macrozamia communis* (34). No Δ5-olefinic acids were reported for these species, but surprisingly, some of them were found to contain cyclopropene acids. Takagi and Itabashi (3) found several Δ5-UPIFA in the seeds of *Cycas revoluta*, though in low amounts, as in *E. sinica* dried stalks.

In the present study, FAME were prepared from *E. nevadensis*, *E. viridis*, *E. przewalskii*, and *E. gerardiana* seed lipids and analyzed by capillary GLC on three fused-silica capillary columns with different polarities, one coated with a 100% cyanopropyl polysiloxane stationary phase (CP-Sil 88; Chrompack, Middelburg, The Netherlands), a second one with a polyethylene glycol stationary phase (DB-Wax; J&W Scientific, Folsom, CA), and a third one with a 1:1-cyanopropyl-phenyl polysiloxane stationary phase (Silar-5CP; Chrompack). Identifications of peaks were performed by comparison of their equivalent chain lengths on the DB-Wax column (isothermal) or their retention times on the CP-Sil 88 and the Silar-5CP columns (temperature programming) with the corresponding parameters of FAME prepared with known sources of individual Δ5-UPIFA. However, owing to the botanical and taxonomic distance between Coniferophytes and Cycadophytes, the absolute FAME structures were also established by GLC–MS of the picolinyl esters prepared from *E. nevadensis* seed lipids.

Additionally, we studied the seed oil from the latter species and from *E. viridis* by ^{13}C nuclear magnetic resonance (NMR) spectroscopy, which allows differentiation of Δ5-UPIFA present in the α and β positions of the triacylglycerols (TAG) because it is known that in all Coniferophyte families analyzed so far, the Δ5-UPIFA are almost completely excluded from the internal position of seed TAG (3,11,12, 35–43). It was therefore interesting to verify whether this structural feature had remained unchanged in the two "branches" of gymnosperms, even after a gap of 300 million years.

MATERIALS AND METHODS

Seeds, oil extraction, and FAME preparation. *Ephedra nevadensis* and *E. viridis* (green jointfir ephedra) seeds were purchased from Lawyer Nursery, Inc. (Plains, MT). Seeds from *E. przewalskii* were collected in the vicinity of Ulan Bator, Mongolia, and seeds from *E. gerardiana* were from the Botanical Garden, University of Hamburg. The extraction of lipids from whole seeds and the preparation of FAME were performed as described in detail elsewhere for other gymnosperm seeds (4,5). All FAME preparations were done in duplicate and analyzed twice on the DB-Wax column and once on the CP-Sil 88 and the Silar-5CP columns. As a rule, FAME were prepared and analyzed on the DB-Wax column immediately after lipid extraction.

Analytical GLC. FAME were analyzed in a Carlo Erba 4130 chromatograph (Carlo Erba, Milano, Italy) equipped with a DB-Wax column (30 m × 0.32 mm i.d., 0.5 μm film; J&W Scientific). The oven temperature was 190°C and the inlet pressure of the carrier gas (helium) was 140 kPa. Alternatively, a CP-Sil 88 column (50 m × 0.25 mm i.d., 0.2 μm film; Chrompack) was operated with temperature programming in a Carlo Erba HRGC chromatograph from 150 to 185°C at 4°C/min with H_2 at 100 kPa. The injector (split mode) and flame-ionization detector were maintained at 250°C for both columns. Quantitative data were calculated by SP 4290 integrators (Spectra Physics, San Jose, CA). Additionally, all FAME samples were analyzed on a Silar-5CP column (Chrompack) using a temperature program standardized for seed oil fatty acid "fingerprints" as described elsewhere (44).

Identification of FAME peaks. The seed lipids from selected conifer species (45) and from Ranunculaceae such as *Cimicifuga* spp. (46) were used as sources of Δ5-olefinic acid methyl esters with known structures to identify fatty acids from *Ephedra* seed lipids by GLC, either by coinjection, comparison of the equivalent chain lengths (DB-Wax column), or retention times (CP-Sil 88 and Silar-5CP). FAME prepared from meadowfoam seed oil were used to locate Δ5-monoenoic (16:1, 18:1, 20:1, and 22:1) acids on chromatograms.

Preparation of picolinyl esters (47). The free acids (1 mg), prepared by hydrolysis with 0.1 M ethanolic potassium hydroxide, were converted to acid chlorides by reaction with oxalyl chloride (0.5 mL) at ambient temperture overnight. Excess reagent was removed in a stream of nitrogen, and the product was reacted immediately with a solution (0.5 mL) of 3-hydroxymethylpyridine in dichloromethane [20 mg/mL; stored over beads of molecular sieve type 4A (Fisher Scien-

tific, Loughborough, United Kingdom)]. After 1 h at ambient temperature, the solvent was evaporated, the product taken in isohexane (5 mL), and washed with water (2 × 2 mL). The isohexane solution was dried over anhydrous sodium sulfate and evaporated; the product was dissolved in isohexane containing butyl-hydroxytoluene (50 ppm) for analysis by GLC–MS.

GLC–MS. The picolinyl ester derivatives were submitted to GLC–MS with a Hewlett-Packard 5890 Series II plus gas chromatograph attached to an HP model 5989 mass spectrometer. The latter was used in the electron impact mode at 70 eV with a source temperature of 250°C. The chromatograph was fitted with on-column injection and equipped with a capillary column of fused silica coated with Supelcowax 10 (25 m × 0.25 mm i.d., 0.25 µm film; Supelco UK, Poole, United Kingdom). After holding the temperature at 80°C for 3 min, the column was temperature programmed at 20°C/min to 160°C, then at 2°C/min to 280°C, where it was held for 5 min. Helium at 1 mL/min was the carrier gas.

^{13}C NMR spectroscopy. ^{13}C NMR spectra were obtained on a Bruker AM 300 spectrometer (Karlsruhe, Germany) operating at a frequency of 75.47 MHz. Samples were prepared in 5-mm tubes with approximately 100 mg of oil (from *E. nevadensis* and *E. viridis* seeds) in 0.5 mL deuteriochloroform that contained tetramethylsilane as reference and $Cr(acac)_3$ as relaxation agent (0.025 M). Spectra were acquired by a nuclear Overhauser effect-suppressed, inverse-gated, proton-decoupling technique and employed a 90° excitation pulse, a 5-s pulse delay, and a sweep width of 20 kHz (32 K data points). The 90° pulse width was 3.9 µs. The number of scans was 1000 per spectrum.

RESULTS AND DISCUSSION

The phyletic position of Ephedraceae. A hypothetical phyletic relationship between Ephedraceae and Conipherophytes is shown in Figure 1 (adapted from Ref. 2). This figure is only intended to help visualize and emphasize the considerable distance between these plant groups on a geological time scale. It is believed that the Gnetatae, to which the Ephedraceae would belong along with the Gnetaceae and the Welwitschiaceae (with only one species, *W. mirabilis*), are the closest living relatives of the angiosperms, though they have common roots with the Coniferophytes (1,26,48). However, the extant Gnetatae might not be the ancestors of angiosperms, because no fossil records of this group have been found before the Tertiary period, while angiosperms certainly existed in the Jurassic (49). Yet, as mentioned above, Δ5-olefinic acids are also present in the seeds of a few rather archaic angiosperm families (22–24).

Fatty acid characterization and composition. Fatty acids are most easily identified by MS in the form of picolinyl esters or 4,4-dimethyloxazoline derivatives (50,51), and the former were used here. Some key diagnostic ions are listed in Table 1. As picolinyl esters, conventional monoenoic and methylene-interrupted dienoic fatty acids are recognized by characteristic gaps of 26 amu in the mass spectra for fragmentation on either side of the double bond and by abundant ions adjacent to the double bond. Picolinyl esters of fatty acids with a 5,9-double bond system can be recognized by a prominent ion at *m/z* = 219, representing cleavage between carbon atoms 7 and 8; other ethylenic bonds are located as for more conventional unsaturated fatty acids (50,51). An isolated double bond in position 5 can be located by the appropriate gap of 26 amu, but a prominent ion at *m/z* = 232 for a fragmentation adjacent to the double bond is often a more useful diagnostic guide. For example, spectra of picolinyl esters of 5-20:1 and 5,13-22:2 acids have been published previously (52). Mass spectra of 4,4-dimethyloxazoline derivatives of the same Δ5-UPIFA prepared from the seed lipids of various conifer species and of *Cimicifuga* spp. have been published previously (45,46).

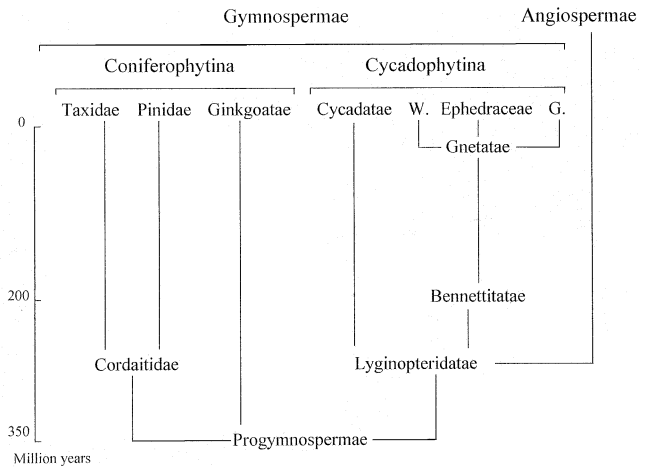

FIG. 1. Hypothetical phylogenic relationship between Ephedraceae and Coniferophytes [W., Welwitschiaceae; G., Gnetataceae) (Adapted from Ref. 2)].

TABLE 1
Characteristic Ions in the Mass Spectra of Picolinyl Ester Derivatives of Unsaturated Fatty Acids in *Ephedra nevadensis* Seed Oil and Their Structures

Fatty acid picolinyl esters	M^+ *m/z* (intensity, %)	Other diagnostic ions *m/z* (intensity, %)
9-16:1	345 (75)	220 (15), 274 (38), 288 (47)
9-17:1	349 (40)	220 (14), 274 (25), 288 (32)
9-18:1	373 (55)	220 (13), 274 (43), 288 (43)
11-18:1	373 (46)	248 (11), 302 (44), 316 (37)
5,9-18:1	371 (22)	219 (25), 260 (12), 272 (10)
5,11-18:1	371 (14)	232 (19), 286 (9), 300 (11)
9,12-18:2	371 (100)	260 (22), 274 (17), 300 (12)
5,9,12-18:3	369 (40)	219 (27), 258 (25), 272 (10)
9,12,15-18:3	369 (77)	300 (39), 314 (14), 340 (17)
5,9,12,15-18:4	367 (17)	219 (16), 258 (14), 298 (14)
11-20:1	401 (45)	248 (10), 302 (30), 316 (23)
13-20:1	401 (33)	276 (10), 330 (28), 344 (23)
5,11-20:2	399 (24)	232 (14), 260 (6), 286 (8)
11,14-20:2	399 (70)	248 (15), 288 (34), 302 (19)
5,11,14-20:3	397 (41)	232 (16), 286 (25), 300 (10)
11,14,17-20:3	397 (36)	368 (10), 328 (24), 288 (14)
5,11,14,17-20:4	395 (31)	232 (15), 286 (17), 326 (21)

With regard to Δ5-UPIFA, all species analyzed here have seed lipids that contain the whole series of such acids (Tables 1 and 2). No Δ5-monoenoic acids were observed on routine chromatograms of FAME (the three capillary columns used here allow distinction of the isomeric *cis*-5 and *cis*-9 18:1 acids; results not shown). With the possible exception of *E. sinica* stalk lipids (3), this appears to be a general feature of gymnosperm lipids. However, in a few species distinct from *Ephedra*, small peaks with the same retention time of 5-18:1 acid have been observed on Silar-5CP columns, which require further investigation and identification (Aitzetmüller, K., unpublished observations). In contrast to most gymnosperm species, Δ5-monoenoic acids have been observed in the seeds of a number of angiosperm families, sometimes in great amounts (22,53), and 5,11,14-20:3 acid also has been observed at levels up to 30% in a few angiosperm seed oils (53).

Our results clearly show that 5,9- and 5,11-18:2 acids, though in minor amounts, coexist in *Ephedra* spp. seed lipids (Table 2), with a higher level of the latter as compared to the former. As dicussed elsewhere for FAME prepared from *G. biloba* seed lipids (13), analyses of FAME prepared from *Ephedra* seed lipids on the DB-Wax capillary column allow only a partial resolution of the 5,9- and 5,11-18:2 acids, whereas baseline resolution of these two isomers is obtained on the CP-Sil 88 and Silar-5CP capillary columns. Identification (by their retention times) and quantitation of these acids were thus made with the latter columns. These identifications were supported by MS of the picolinyl esters separated on a Supelcowax capillary column (similar to the DB-Wax) (Table 1).

The same octadecadienoic acids that were formally identified in *G. biloba* seeds (13) were identified by GLC–MS of the picolinyl esters, and our observations confirm the tentative identifications of these acids by Takagi and Itabashi (3) in *E. sinica* dried stalks, based on calculated equivalent chain lengths. In the study by Kleiman *et al*. (27) on *E. campylopoda* seed lipids, only the 5,11-18:2 isomer was identified by chemical means. For practical purposes, it would appear that *Ephedra* seeds are more useful than *G. biloba* seeds in preparing 5,11-18:2 acid, because their oil content is *ca*. 11%

TABLE 2
Fatty Acid Composition (wt% of total fatty acids[a]) of Four *Ephedra* spp. Seed Lipids (this study) and Comparison with *E. campylopoda*

Fatty acid structure[b]	Species				
	E. nevadensis	*E. viridis*	*E. przewalskii*	*E. gerardiana*	*E. campylopoda*
14:0	0.1	0.1	0.7	0.1	Trace
16:0	6.1	6.2	6.9	6.6	7.2
7-16:1	0.2	0.2	0.5	0.1	
9-16:1	0.9	0.4	0.7	0.7	0.4
aiso-17:0	Trace	Trace	0.2	—[c]	—
17:0	0.03	0.03	0.2	0.1	—
9-17:1	0.04	0.03	0.1	Trace	—
18:0	2.3	2.5	3.4	2.5	7.9
9-18:1	34.2	29.2	24.6	17.1	26.8
11-18:1	12.2	9.7	7.3	11.2	6.2
9,12-18:2	10.6	13.5	9.3	8.7	9.3
9,12,15-18:3	7.9	9.5	16.8	10.5	7.8
20:0	0.3	0.3	0.5	0.4	0.1
11-20:1	0.4	0.5	0.6	0.5	0.1
13-20:1	0.3	0.22	0.2	0.5	0.1
11,14-20:2	1.2	2.0	0.7	2.6	1.4
7,11,14-20:3	Trace	Trace	—	—	—
11,14,17-20:3	0.9	1.5	1.5	3.3	2.2
22:0	0.2	0.2	0.3	0.2	—
5,9-18:2[d]	1.4	1.0	0.5	0.4	—
5,11-18:2[d]	4.0	1.9	2.0	1.7	2.0
5,9,12-18:3	0.1	0.2	Trace	Trace	—
5,9,12,15-18:4	0.2	0.2	0.5	0.5	—
5,11-20:2	1.6	1.5	1.1	1.5	1.2
5,11,14-20:3	5.6	6.6	4.4	7.5	5.4
5,11,14,17-20:4	8.9	11.7	8.8	19.2	21.9
Others	0.4	0.8	8.2[e]	4.2[e]	0.0
Σ Δ5[f]	21.9	23.1	17.3	30.8	30.5

[a]Means of analyses of two methyl ester preparations.
[b]Double bonds are counted from the carboxylic group and are in the *cis* configuration.
[c]Not reported (Ref. 27), or not detected (present study).
[d]Proportions determined on the CP-Sil 88 or the Silar-5CP columns.
[e]May contain some unsaponifiable components (late-eluting peaks).
[f]Sum of Δ5-olefinic acids.

(relative to undehulled seeds) instead of only 1.5–2%, respectively. The 5,9,12-18:3 and 5,9,12,15-18:4 acids, which are the Δ5-desaturation products of linoleic and α-linolenic acids, are present in small amounts (0.1–0.5%), and were not reported previously in *Ephedra* seeds (27,28). However, an isomeric 18:3 acid (unknown structure) was noted on the chromatogram of FAME prepared from *E. nevadensis*, and published by Litchfield (30). The 5,11-20:2 acid (derived from 11-20:1 acid) also occurs in the seed lipids of the four *Ephedra* species (1.1–1.6%), but we could not detect the Δ5-desaturation product of 13-20:1 acid, the elongation product of *cis*-vaccenic acid. Both the 9,12-18:2 and the 9,12,15-18:3 acids are elongated to 11,14-20:2 and 11,14,17-20:3 acids, respectively, but none of these acids accumulated to a large extent (3.3% at most). They are apparently actively Δ5-desaturated to 5,11,14-20:3 and 5,11,14,17-20:4 acids, which are present at levels in the range 4.4–7.5% and 8.8–19.2%, respectively. The total Δ5-UPIFA reached 17.3% in *E. przewalskii*, 21.9% in *E. nevadensis*, 23.1% in *E. viridis*, and 30.8% in *E. gerardiana*. The latter value compares well with the proportion determined in *E. campylopoda* (30.5%) (Table 2). It is interesting to note that total Δ5-UPIFA is less than 33% in the five *Ephedra* species analyzed so far, such as the Conipherophyte species. For the latter species, this was explained by the fact that all Δ5-UPIFA are mostly esterified to only one position of TAG, the *sn*-3 position (37).

Among "habitual" fatty acids, the main component was oleic (9-18:1) acid (17–34%), accompanied by fairly high levels of 11-18:1 (*cis*-vaccenic or asclepic) acid (*ca.* 7–12%). Among Coniferophytes, only *G. biloba* seeds present a higher level of *cis*-vaccenic acid (17–18%) (3,54,55); other species generally contain less than 2% of this acid in their seed lipids. Following the octadecenoic acids, and in more or less equal abundance, are linoleic (9,12-18:2) and α-linolenic (9,12,15-18:3) acids (9–13% and 8–17%, respectively). The level of α-linolenic acid was between that present in Pinaceae and Taxaceae on the one hand, and that in Taxodiaceae and Cupressaceae on the other hand. The saturated fatty acids, principally 16:0 and 18:0 acids, were quite low (less than 10% in the species analyzed here), which is an habitual feature of most Coniferophytes. Other minor fatty acids were 14:0, possibly 15:0, 17:0, 9-17:1 [identified by its mass spectrum (Table 1)], and 22:0 acids. 14-Methylhexadecanoic acid, which is an ubiquitous branched fatty acid in Pinaceae seeds (56), and also present in *G. biloba* seeds (55), could not be positively identified in *Ephedra* seeds.

Hypotheses on Δ5-desaturases in gymnosperms. A metabolic pathway for the synthesis of habitual fatty acids and of Δ5-UPIFA was proposed initially by Itabashi and Takagi (57), who analyzed the fatty acid composition of *Taxus cuspidata* (Japanese yew) seed, aril, and leaf lipids. Later, Wolff *et al.* (5) proposed a similar scheme based on the analysis of the seed fatty acids from 28 species belonging to the families Pinaceae, Taxaceae, Taxodiaceae, Sciadopityaceae, and Cupressaceae. Both groups of researchers postulated the existence of a Δ5-desaturase that would use 9-18:1, 9,12-18:2, 9,12,15-18:3, and their elongation products, 11-20:1, 11,14-20:2, and 11,14,17-20:3 acids, as substrates. Additionally, in *G. biloba* seed lipids (13) and in *Ephedra* spp. (this study), the 11-18:1 acid also is visibly a substrate for the Δ5-desaturase and gives 5,11-18:2 acid. With regard to this acid, we propose the trivial name "ephedrenic" acid, to recall that it is a widespread fatty acid in *Ephedra* spp. seeds, though it also occurs in *G. biloba* seed lipids.

Our observations suggest that there might exist two Δ5-desaturases in Coniferophyte and Ephedraceae seeds, one specific for unsaturated fatty acids with a Δ11-ethylenic bond, and another one specific for unsaturated fatty acids with a Δ9 double bond (Figure 2). In the particular case of *Ephedra* spp. seeds, the former would present a high activity, whereas the latter would be considerably less active: Fatty acids with a 5,11-ethylenic pattern are considerably higher than those containing a 5,9 system. For example, an opposite situation is observed in Pinaceae and in some Taxaceae [e.g., *Taxus* species (11)], where the 5,9-system is generally prominent. *Pinus edulis* (9) and other pines from the subsection *Cembroides* (10) are exceptions. In these species, the proportion of 5,11,14-20:3 acid may be higher than that of the 5,9,12-18:3 acid, though both acids are exceptionally low in this subsection of *Pinus*. On the other hand, the 5,11-dienoic arrangement predominates in some other families such as Sciadopityaceae [with the single extant species *Sciadopitys verticillata* (58)], Cupressaceae, and Taxodiaceae (3,5,7,14). If one ignores the existence of the 5,11-18:2 acid in *G. biloba* and *Ephedra* spp. seeds, then one can also hypothesize that there may exist two desaturases, one specific for unsaturated C_{18} acids, and the other specific for unsaturated C_{20} acids (Fig. 2). A third possibility—at least in some Cycadophytes—would depend on different chain-elongation activities (from C_{18} to C_{20}), prior to Δ5-desaturation by only one Δ5-desaturase, which generally prefers Δ11- and/or C_{20} substrates over Δ9- and/or C_{18} substrates. This model could also explain the observed different Δ5-UPIFA levels. The Δ5-UPIFA would then simply depend on the relative amounts of the preformed Δ11- (e.g., 11-18:1) or C_{20} acid substrates that were available to attack a single Δ5-desaturase.

The problem in making a decision regarding these possibilities is linked to the existence of 5,11-18:2 acid, issued from 11-18:1 acid, which is both a C_{18} acid and a Δ11-unsaturated acid. Moreover, the 13-20:1 acid, a C_{20} monoenoic acid without a Δ11-ethylenic bond, is apparently not a substrate for the Δ5-desaturase(s). These two exceptions would favor the first hypothesis, i.e., a specificity of the Δ5-desaturases for either the Δ9- or the Δ-11 ethylenic bond. In view of the fact that no Δ5-monoenoic acids are present, one ethylenic bond in the latter positions is apparently necessary for the introduction of a Δ5-ethylenic bond. Whatever the number of Δ5-desaturases and their substrate specificities, it is likely that they exist in both "branches" of gymnosperms. This is clearly demonstrated for *Ephedra* spp. and *Cycas revoluta* (3), another Cycadophyte. Except for the elongation of 5,9,12-18:3 acid to 7,11,14-20:3 (bishomopinolenic) acid,

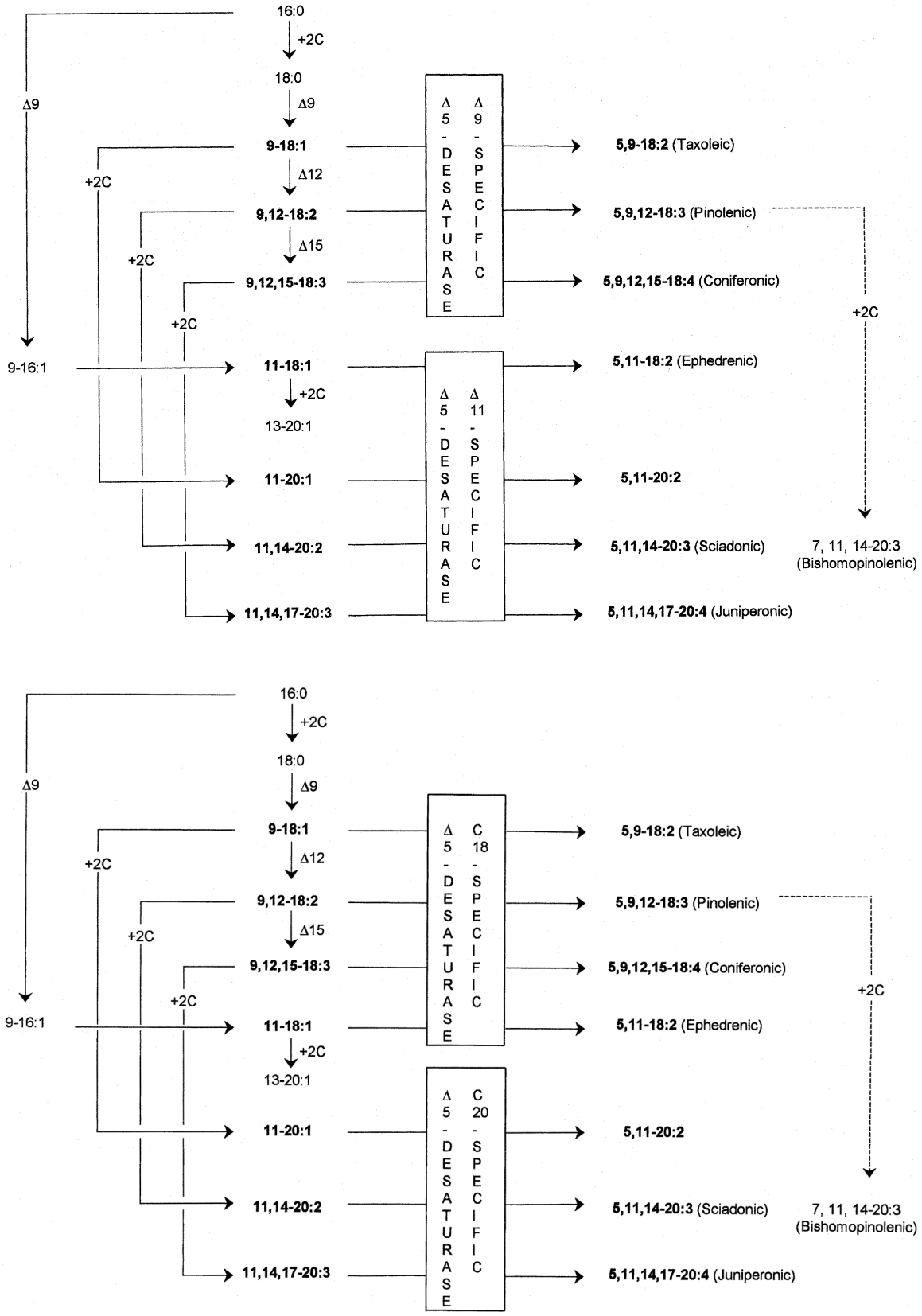

FIG. 2. Possible metabolic pathways for the biosynthesis of known unsaturated fatty acids occurring in gymnosperm seeds, and particularly in *Ephedra* spp. seeds. The upper figure is based on a specificity of Δ5-desaturases for the first ethylenic bond (Δ9 vs. Δ11), and the lower figure is based on a specificity for the chain length (C_{18} vs. C_{20}). The reaction symbolized by a dotted arrow is apparently limited to Pinaceae species, and does not seem to take place in *Ephedra* spp. seeds.

which was shown to occur in many species, but exclusively in the Pinaceae family (59), all other reactions shown in Figure 2 would occur simultaneously in *Ephedra* spp. seeds. The end products as well as the intermediate fatty acids have all been characterized in the present study.

When considering the ancestors of gymnosperms, possibly an extinct group of Pteridophytes (or derived from them, e.g., the "seed ferns"), or some still more remote ancestors, it is interesting to note that some extant species of the latter plant group (e.g., ferns) can synthesize arachidonic (5,8,11,14-20:4) and eicosapentaenoic (5,8,11,14,17-20:5) acids in their green parts (29). This implies that they contain one Δ6-desaturase specific for polyunsaturated C_{18} acids and one Δ5-desaturase specific for polyunsaturated C_{20} acids, which is a feature common to the more ancient group Bryophytes [e.g., mosses (29,60,61) and liverworts (61)], many microalgae and algae, some molds, and to most animals. However, the simultaneous presence of both Δ5- and Δ6-acids also has recently been observed in an angiosperm at levels between 3 and 5% of each (Aitzetmüller, K., unpublished results). The passage from Pteridophytes (or a related plant group) to gymnosperms might thus have been accompanied by a change of the C_{18}-specific (or Δ9-specific) Δ6-desaturase to a C_{18}-specific (or Δ9-specific) Δ5-desaturase, whereas the C_{20}-specific (or Δ11-specific) Δ5-desaturase would have kept its specificity. No fatty acids with a Δ6-ethylenic bond have been reported in any of the gymnosperm families analyzed so far, but conclusive results supported by MS indicate that the seeds from at least one species of the Araucariaceae family would contain both the Δ6-desaturase specific for polyunsaturated C_{18} acids and the Δ5-desaturase specific for polyunsaturated C_{20} acids. Their lipids contain γ-linolenic, stearidonic, arachidonic, and eicosapentaenoic acids, in addition to C_{20} Δ5-olefinic acids characteristic of other Coniferophytes (Wolff, R.L., W.W. Christie, F. Pédrono, and A.M. Marpeau, unpublished results). Because the interfamily relationships of Araucariaceae among Conipherophytes are mostly obscure, and because little is known as to which family could be more primitive, it is unclear whether the original status of polyunsaturated fatty acids in gymnosperms was a Δ6-unsaturated C_{18} status or a Δ5-unsaturated C_{18} status.

The possibility of the simultaneous presence of a C_{18}-specific Δ6-desaturase and of a C_{20}-specific Δ5-desaturase in a Spermaphyte is more than of academic interest. The logical metabolites of the combined action of both desaturases, with the intermediate intervention of a C_{18}-C_{20} elongase, are arachidonic and eicosapentaenoic acids. Surprisingly enough, arachidonic acid has been characterized in a few rare angiosperm species that are not known to contain the metabolic precursor γ-linolenic acid, whereas species containing the latter acid would not contain arachidonic acid (62). It is noteworthy that garlic and virgin wheat germ oil, have been reported to contain arachidonic acid (62).

Distribution of Δ5-UPIFA between the α- and β-chains of TAG. Several studies, using either chemical or enzymatic degradative procedures (3,36–39,43) or ^{13}C NMR spectroscopy (11,12,35,36,40–43), have shown that in all Coniferophyte seed lipids analyzed so far (more than 30 species belonging to 8 families and 15 genera; Table 3), Δ5-UPIFA are practically excluded from the β-position of TAG. This was corroborated by two studies, indicating that TAG molecular species with two Δ5-UPIFA were scarce [*Pinus pinaster* and *P. koraiensis* (63)] or undetectable [*P. koraiensis* (64)]. A more detailed study of the stereodistribution of Δ5-UPIFA has shown that they were principally esterified (more than 90%) to the *sn*-3 position [in *Taxus baccata*, *Larix decidua*, *Sciadopitys verticillata*, *Juniperus communis*, and the two preceding *Pinus* species (37)]. This results in a maximum observed content of Δ5-UPIFA in TAG of 33% of total fatty acids [in *Larix sibirica* seeds (65)], supporting the hypothesis that these acids would be exclusively esterified to the *sn*-3 position of TAG from

TABLE 3
Coniferophyte Species Showing a Definite Enrichment of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acids in the α-Chains of Seed Triacylglycerols

Family	Species	Methods[a]	References[b]
Ginkgoaceae	*Ginkgo biloba*	GR	(3)
Podocarpaceae	*Podocarpus nagi*	GR	(3)
	P. andinus	NMR	(12)
Taxaceae	*Torreya nucifera*	GR	(3)
	T. grandis	NMR	(11)
	Taxus cuspidata	GR	(3)
	T. baccata	GR, NMR	(35–37)
	T. chinensis	NMR	(11)
Cephalotaxaceae	*Cephalotaxus drupaceae*	NMR	(12)
Pinaceae	*Pinus armandi*	—	(38)
	P. koraiensis	GR, NMR	(3,35–39)
	P. cembra	-—	(38)
	P. sylvestris	NMR	(35,40)
	P. mughus	NMR	(35)
	P. nigra	NMR	(35)
	P. griffithii	NMR	(35)
	P. pinaster	GR, NMR	(35–37)
	P. pinea	GR	(36)
	Larix decidua	GR, NMR	(36,37)
	L. leptolepis	NMR	(41)
	L. sibirica	NMR	U.R.[c]
	Picea jezoensis	GR	(3)
	P. abies	NMR	(41)
	P. sitchensis	NMR	(41)
	Cedrus atlantica	NMR	(41)
	Abies concolor	NMR	(41)
	A. alba	NMR	(42)
Sciadopityaceae	*Sciadopitys verticillata*[d]	GR, NMR	(36,37,41)
Taxodiaceae	*Cryptomeria japonica*	GR	(3)
Cupressaceae	*Thuja occidentalis*	NMR	(41)
	Juniperus virginiana	NMR	(41)
	J. communis	GR	(36,38)
	Biota orientalis	PL, NMR	(43)

[a]GR, Grignard reagent; NMR, ^{13}C-nuclear magnetic resonance spectroscopy; PL, pancreatic lipase.
[b]References 3, 36, 37, 38, and 43 relate to purified triacylglycerols; data in Refs. 3, 11, 35, 40, and 41 were determined with total lipids, and those in Reference 42 were established with whole seeds. Reference 38, and experimental procedures therein, are in Japanese.
[c]Farines, M., and Wolff, R.L., unpublished results.
[d]Erroneously reported earlier as a species of the Taxodiaceae family (58).

Coniferophyte seeds.

One aim of the present study was to determine whether the systematic and specific esterification of Δ5-UPIFA to the *sn*-3 glycerol hydroxyl observed in the Coniferophyte branch also occurred in the Cycadophyte branch, even after a separation of 300 million years. As a first approach, ^{13}C NMR spectroscopy was applied to *E. nevadensis* and *E. viridis* seed crude oils. As detailed elsewhere (35,41), this technique not only allows calculation of Δ5-acid levels, but also distinction of Δ5 acids esterified to the α (*sn*-1/3) and β (*sn*-2) positions of TAG with specific reference to carbon atoms 1 (acyl) and 2. The *E. nevadensis* oil has three signals for both C_1 and C_2 corresponding to Δ5 acids in the α position and to the remaining acids in the α and β positions. *Ephedra viridis* oil shows the same major signals accompanied by some small signals which cannot be assigned with certainty. One of these may relate to Δ5 acids in the β position. The results are set out in Table 4. The levels of Δ5 acids measured by NMR (mol%)—both C_1 and C_2 signals—are in good agreement with the more accurate levels measured by GLC (wt%). Consequently, the Δ5 acids occur wholly or mainly in one or both of the α positions in *Ephedra* spp. seed TAG, and the level of these acids in the β position does not exceed 3% (experimental limit detection level).

Because ^{13}C NMR spectroscopy does not distinguish between the *sn*-1 and *sn*-3 positions, our conclusion is thus limited to the α positions. At this molecular level, Coniferophytes and Cycadophytes (at least those species containing Δ5-UPIFA) share in common the distribution of Δ5 acids in TAG. The present study provides some evidence that the unusual role for Δ5 unsaturation [or of the Δ5-desaturase(s)] in the acylation (or desaturation) of glycerol esters in gymnosperm seed lipids is of great antiquity. Apparently, the stereospecific distribution of Δ5-UPIFA in the seed TAG from species of the two gymnosperm branches has remained unchanged during the past 300 million years.

Few data are available for the intraglyceride distribution of Δ5-UPIFA in angiosperm species presenting such acids in their seed lipids. The best documented species are apparently *Limnanthes* species, i.e., *L. douglasii* (66) and *L. alba* (67). The former species was subjected to complete stereospecific analysis, and it was observed that Δ5-olefinic acids (mostly 5-20:1, 5-22-1, and 5,13-22:2 acids, none of which occur in gymnosperm seeds) were esterified to all three glyceride positions, although not in equal amounts. Δ5-Olefinic acids were more abundant in the α positions (almost equally distributed between the *sn*-1 and *sn*-3-positions) than in the β position (66). The TAG species in *L. alba* seeds have been investigated, and almost 51% of them contained three Δ5-olefinic acids (67).

From the absence of 5-18:1 and 5-20:1 acids in reports on gymnosperm seed lipids (which is a distinctive feature as compared to some angiosperms containing Δ5 acids), it may be inferred that the Δ5-desaturase(s) acts as a final step in the biosynthesis of C_{18} Δ5-UPIFA, that is after the introduction of Δ9-, Δ12-, and Δ15-ethylenic bonds, and of the C_{20} Δ5-UPIFA after elongation of oleic, linoleic, and α-linolenic to the corresponding bishomo derivatives. However, it cannot be decided whether the introduction of the Δ5-ethylenic bond occurs before or after incorporation of C_{18} and C_{20} unsaturated acids into TAG. In both cases, one should postulate the existence of either Δ5-desaturase(s) or an acylase specific for the *sn*-3 position of glycerol. This emphasizes some major differences between gymnosperms and angiosperms with regard to the biosynthesis of Δ5-acylated TAG. Within the Gnetatae, the Ephedraceae seed TAG composition and structure are gymnospermous biochemical characteristics apparently not shared by Gnetaceae and Welwitschiaceae (48), which may help clarify the much controversed taxonomy and phylogeny of Gnetatae (1).

We also have found a different tentative classification of gymnosperms by Meyen (68). This author suggests another taxonomic and phyletic position for the family Ephedraceae, which he includes in the order Ephedrales. This order is grouped with Ginkgoales and seven other extinct orders in the class Ginkgoopsida. The reason for this grouping is the primary platyspermy of the seeds, possibly "of utmost importance for tracing gymnosperm phylogeny" (1). Coincidentally, *Ephedra* (this study) and *Ginkgo biloba* (13) are the two single genera among the 170 gymnosperm species we analyzed that contain both taxoleic and ephedrenic acids in sig-

TABLE 4
Total Content of Δ5 Acids in the Seed Oil from Two *Ephedra* Species as Determined by ^{13}C NMR Spectroscopy and by GLC, and Their Content in the α and β Positions[a]

		Fatty acid content[b]					
		α and β positions				Total	
Species	Signal	Δ5 (α)[c]	Δ5 (β)	Other (α)	Other (β)	Δ5 (NMR)	Δ5 (GLC)
Ephedra nevadensis	C1	23.9	—	40.9	35.2	23.9	21.9
	C2	23.4	—	43.6	33.0	23.4	21.9
E. viridis	C1	25.9	2.5[d]	36.3	35.3	25.9 (28.4)[e]	23.1
	C2[f]	23.6	2.6[d]	40.5	31.5	23.6 (26.2)	23.1

[a]NMR, nuclear magnetic resonance; GLC, gas–liquid chromatography.
[b]NMR data, mol%; GLC data, wt%.
[c]Total Δ5 acids at the indicated position.
[d]Uncertain assignments.
[e]Total values in parenthesis include the uncertain 2.5/2.6%.
[f]Also an unassigned signal of 1.8%.

nificant amounts.

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