pattern, infrared spectra, and a photomicrograph, respectively.

LITERATURE CITED

Akiyama, T.; Ando, J. *Bull. Chem. Soc. Jpn.* **1972**, *45* (9), 2915-2920.
Potts, J. M.; Rindt, D. W.; Slack, A. V. *Chem. Eng. Prog.* **1962**, *58* (9), 89-93.
Tennessee Valley Authority, National Fertilizer Development Center, Muscle Shoals, AL, 1978, Bulletin Y-136.
Vidzhesingkh, B.; Kuznetsova, A. G.; Torocheshnikov, N. S.; Kozlova, G. A. *J. Appl. Chem. USSR (Engl. Transl.)* **1976**, *49* (2), 2074-2075.

Received for review August 4, 1980. Accepted December 1, 1980.

Fatty Acids and Sterols Associated with Citrus Root Mycorrhizae

Harold E. Nordby,* Stanley Nemec, and Steven Nagy

The structures of four fatty acids present in citrus roots infected with VA-mycorrhizal fungus but not in noninfected roots were shown to be 11*c*-16:1, 6*c*,9*c*,12*c*-18:3, 8*c*,11*c*,14*c*-20:3, and 5*c*,8*c*,11*c*,14*c*-20:4. Compositional differences of these four acids between seedlings infected with three *Glomus* species were slight. These acids were present in the triglycerides and to a lesser extent in three phospholipids and one glycolipid of the fibrous roots. They were absent in the tap roots and leaves. Supplemental phosphorus failed to produce these acids in seedlings grown in noninoculated soil. Levels of these acids were lower in mature trees than in seedlings. Lipids from *Glomus mosseae* chlamydospores contained 11*c*-16:1 as well as a 20:5 fatty acid and almost exclusively a 24-methylcholesterol in its free sterol fraction.

Mycorrhizal fungi have a growth-stimulating effect on citrus seedlings (Marx et al., 1971; Kleinschmidt and Gerdemann, 1972; Nemec, 1978) by enhancing nutrient uptake (Gerdemann, 1968). When these endomycorrhizal fungi are killed by heat treating or fumigating the soil in which the citrus seedlings are grown, stunting and chlorosis of the plants result. These effects are most pronounced in phosphorus-deficient soils. Lipids have been shown by microscopic examination (Cox and Sanders, 1974; Cox et al., 1975) and by solvent extraction (Cooper and Losel, 1978) to be more abundant in mycorrhizal-infected than in noninfected fibrous roots. When labeled lipid precursers were fed to onion seedlings, greater amounts of the label were observed in the infected roots (Losel and Cooper, 1979). Many hypotheses have been made as to the role lipids play in this growth stimulation (Cox et al., 1975; Harley, 1975). Citrus roots infected with *Glomus mosseae* were shown to contain three fatty acids that are unusual to higher plant material and which constituted over 40% of the total fatty acids (Nagy et al., 1980); a fourth unusual acid not reported with those three was present at less than 0.5%. These four acids, however, were not detected in control citrus roots. The acids were concentrated to the greatest extent (46-52%) in triglycerides but were also found in the glyco-polar lipids (7-20%). Also significant was the finding that campesterol/free demethyl sterol ratios were higher for the infected citrus roots than for the noninfected. For possible clarification of the role lipids, and specifically the role these unusual fatty acids play in this growth-stimulating effect of mycorrhizal fungi, a series of experiments were designed to answer the following questions: (1) What are the structures of these unusual fatty acids? (2) Are these acids, as well as sterols, present in the fungus itself? (3) Are these acids found in roots infected with mycorrhizal fungi other than *G. mosseae*? (4) Are these acids found in roots of citrus seedlings grown in sterile soil supplemented with superphosphate to the extent that the growth of the seedlings is comparable to that of the infected seedlings? (5) Are these acids limited to the arbuscules, vesicles, and intercellular and external hyphae regions of the fibrous roots (Cox and Sanders, 1974; Cox et al., 1975) or may they be present in other tissues, such as the tap roots or leaves of the infected plant? (6) Are these acids preferentially found in certain phospholipids or glycolipids? (7) Are these acids found in roots of citrus plants in later stages of growth, e.g., 2 and 9 years of age?

U.S. Citrus and Subtropical Products Laboratory, Southern Region, U.S. Department of Agriculture, Science and Education Administration, Winter Haven, Florida 33880 (H.E.N.), U.S. Horticultural Field Station, Southern Region, U.S. Department of Agriculture, Science and Education Administration, Orlando, Florida 32802 (S. Nemec), and Florida Department of Citrus, Lake Alfred, Florida 33850 (S. Nagy).

EXPERIMENTAL SECTION

Preparation of Plant and Fungal Material. *Experiment 1.* Lipids from which concentrates of the four unusual fatty acids were prepared were from our first study (Nagy et al., 1980) and from tissues obtained from experiments 3-7 of this study.

Experiment 2. Individual chlamydospores of *G. mosseae* from the Sudan grass host (Nemec, 1978) were removed under microscopic examination with tweezers and stored in a bottle of ethanol-benzene (1:4 v/v) under refrigeration.

Experiment 3. The three rootstocks used in this study were Rangpur lime (*Citrus reticulata* var. austera Swing.), sweet orange (*Citrus sinensis* L. Osb.), and sour orange (*Citrus aurantium* L.). Plants were germinated in a greenhouse from seeds planted in flats of steamed Astatula fine sand subsoil low in phosphorus (<20 ppm). Methods for fertilization, inoculation with *Glomus fasciculatus*, and transplantation were previously detailed (Nemec, 1978). The seedlings were 186 days old when the fibrous roots were excised.

Experiment 4. Sour orange plants were grown as in experiment 3 but were inoculated with *Glomus etunicatus*. The noninoculated citrus plants were grown in soil en-

riched with superphosphate equivalent to 2240 kg of triple superphosphate/ha. Seedlings were 200 days old when the fibrous roots were excised.

Experiment 5a. Rough lemon (*Citrus limon*, L. Burm. F.) were grown as seedlings as in experiment 3, with the soil of the experimental plants inoculated with *G. mosseae*. Plants were excised after 230 days, washed, blotted dry, and separated into fibrous roots, tap roots, and leaves (minus the stem).

Experiment 5b. Rough lemon seedlings were grown for 133 days in noninoculated soil and soil inoculated with *G. etunicatus*. The fibrous roots were carefully removed from the soil, washed, and blotted dry.

In experiments 3, 4, and 5a, four samples of each rootstock were taken from control (C) and mycorrhizal (M) plants for lipid extraction; in experiment 5b, three samples each were obtained.

Experiment 6. The samples of lipids used for the phospholipid study were from the extracts obtained from the sour orange seedlings in experiment 4 and from the rough lemon seedlings in experiment 5a. the lipids for the glycolipid study were from experiment 5b.

Experiment 7. For this study, 2-year-old Carrizo citrange [*Poncirus trifoliata* (L.) Raf. × *C. sinensis* (L.) Osbeck] grown as liners for budding and a 9-year-old seedling Duncan grapefruit (*Citrus paradisi* Macf.) grown in the variety collection at the Leesburg farm, USDA, Leesburg, Fl, were used. Both of the rootstocks were grown under normal citrus horticultural conditions, the soil having been fumigated with methyl bromide prior to the plants being set out in the field.

Lipid Extraction, Fractionation, and Ester Preparation. *Experiment 2.* The benzene–ethanol solvent containing the *G. mosseae* chlamydospores was removed on a rotoevaporator in vacuo under nitrogen at 30 °C. The spores (~4 mg) were extracted in an Elvehjem tube with $CHCl_3$–MeOH (2:1 v/v) (Folch reagent) over a 2-h period at room temperature. Lipids were recovered in $CHCl_3$, washed with water, and dried over Na_2SO_4. The lipid was used for lipid fractionation, fatty acid preparation, and sterol isolation.

Experiments 3, 4, 5, and 7. Finely cut fibrous roots, tap roots, or leaves (1.5–3.0 g) were extracted as reported previously (Nagy et al., 1980) with Folch reagent. The extracts were combined and concentrated in vacuo under nitrogen at 30 °C.

For separation of free sterols, triglycerides, and glyco-polar lipids, a portion of the total lipids was subjected to preparative thin-layer chromatography (TLC) on silica gel G plates with chloroform as the developing solvent. The air-dried plates were sprayed with Rhodamine 6G and viewed under UV light. Triglycerides (R_f 0.76) and demethyl sterols (R_f 0.30) were resolved from the coumarins (R_f 0.50), which moved as a wide band by this TLC system. The entire area between the origin and the demethyl sterol band was designated "glyco-polar lipids".

For the isolation of the three major phospholipids in experiment 6, portions of the total lipid extracts were streaked on two 250-μm silica gel G plates. The first plate was developed in chloroform–methanol–acetic acid–water (170:25:25:6 v/v/v/v) and the second plate in chloroform–methanol–7 N ammonium hydroxide (130:60:8 v/v/v). A 2-cm strip on both sides of each plate was sprayed with a phosphate spray (Dittmer and Lester, 1964). Three blue areas corresponding to cochromatographed standards of phosphatidic acid, phosphatidylinositol, and phosphatidylcholine were observed on both plates. The nonsprayed portions of the plate corresponding to these three phospholipids were scraped from the plate for fatty acid ester preparations.

For preparation of a glycolipid fraction, a portion of the lipid from experiment 5b was placed on a silica gel column, and the three fractions were eluted with 200 mL of chloroform (neutral lipids), 200 mL of acetone (glycolipids) and 200 mL of methanol (polar lipids). The glycolipid fraction was plated on 250-μm silica gel G plates and developed in chloroform–methanol (170:30 v/v) to obtain the major glycolipid, esterified steryl glucoside (ESG).

Fatty acid methyl esters (FAME's) from total lipids, triglycerides, glyco-polar lipids, esterified sterol glucosides, and the three phospholipids were prepared with NaOH–BCl_3 (Peterson et al., 1965). Except for the total lipid preparations, the FAME's were prepared from the lipid still on the TLC silica gel adsorbent. All FAME's were purified by TLC on silica gel G plates with hexane–ethyl (9:1) as solvent. Sterols were removed from the TLC plate in experiment 2 by elution of the silica gel with ethyl ether.

Fatty Acid and Sterol Quantitations. FAMEs were determined on a 1.52 m × 4 mm i.d. glass column with 3% SP-1000 on 100–120 Gas-Chrom Q. Injection port and detector were at 250 °C, and the helium flow was 60 mL/min. On-column injections of the FAME's in hexane were run isothermally at 158 °C. Free demethyl sterols were run isothermally on 1.52 m × 4 mm i.d. glass columns with 1% SP-1000 on 100–120 Gas-Chrom Q and with 1% Dexsil 300 on 100–120 Gas-Chrom Q at 205 and 220 °C, respectively, and a helium flow of 60 mL/min for both columns. Peak areas were measured with the aid of an Autolab Systems IV electronic integrator. Values reported for these classes of compounds are the means of duplicate analyses on two to five replicates. For simplification of this presentation, values for the "nonmycorrhizal fatty acids" are combined into two groups in Table III; iso-16:0, 16:0, 9*c*-16:1, and anteiso-17:0 (∑16's and 17's), and 18:0, 18:1, 18:2 and 18:3 (∑18's).

Fatty Acid Structure Determinations. Gas–liquid chromatographic retention times and equivalent chain lengths (ECL's) of the standard FAME's and the isolated fatty acid methyl esters were determined on the above-described 3% SP-1000 column and on the following 1.52 m × 4 mm i.d. glass columns: 3% SE-30 on Chromosorb W, 80–100, at 180 °C, 10% DEGS on Gas-Chrom Q, 100–120, at 170 °C, and 10% Apolar 10C on Gas-Chrom Q, 100–120, at 180 °C. The helium flow on all columns was 60 mL/min. Portions of the FAME's in hexane were hydrogenated in a Parr apparatus with Pd/C for 1 h at 60 psi (Nordby and Nagy, 1969). Silver nitrate TLC separation of the FAME's according to their degree of unsaturation were performed on 250-μm silica gel G plates (Nordby and Nagy, 1969). The C-16 and C-18 monoenes were analyzed by GC–MS as pyrrolidides (Andersson and Holman, 1973) and as ozonides of the methyl esters by chemical ionization GC–MS at the Hormel Institute, University of Minnesota, Austin, MN. Concentrates of the standards, *trans*-3-hexadecenoic methyl ester and fungisterol, were isolated from fresh spinach and oats, respectively. Methyl stearolate was obtained courtesy of the Northern Regional Research Center, SEA, USDA. Standard γ-linolenic acid methyl ester, 8,11,14-eicosatrienoic acid methyl ester, and 5,8,11,14-eisosatetraenoic acid methyl ester (methyl arachidonate) were purchased from Altech Associates, Arlington Heights, IL. Synthetic 11*c*-16:1 fatty acid was obtained from the University of Pennsylvania.

RESULTS

Structures of "Mycorrhizal Fatty Acids". ECL

Table I. Effective Chain Lengths (ECL's) of C-16 to C-20 Fatty Acids Associated with Endomycorrhizae

fatty acid[a]	source[b]			ECL on GLC liquid phase[c]			
	I	S	C	SE-30	SP-1000	DEGS	Apolar 10C
3*t*-16:1		X		16.0	16.5	16.7	16.7
9*c*-16:1	X	X		15.7	16.2	16.5	16.8
11*c*-16:1	X	X		15.9	16.4	16.7	17.0
9*c*-18:1	X[d]	X		17.7	18.2	18.5	18.8
9a-18:1		X		18.0	20.4	20.3	18.5
6,9,12-18:3	X	X			19.0	19.8	20.7
9,12,15-18:3	X	X		17.4	19.3	20.3	21.1
5,8,11-20:3			X		20.6	21.5	22.3
8,11,14-20:3	X	X		19.4	20.9	21.9	22.8
8,11,14-20:3			X		20.8	21.8	22.5
11,14,17-20:3			X		21.2	22.2	23.0
5,8,11,14-20:4	X	X		19.1	21.1	22.4	23.3
5,8,11,14-20:4			X		21.0	22.2	23.1
8,11,14,17-20:4			X		21.4	22.8	23.8
5,8,11,14,17-20:5	X				21.5		24.4
5,8,11,14,17-20:5			X		21.6		24.4

[a] Number and positions of double bonds; *t* = trans; *c* = cis; a = acetylenic; polyenes are all cis. [b] I = isolated from citrus roots or spores; S = standard; C = calculated from the formula $ECL_x = aECL_{18:3} + b$ (Jamieson and Reid, 1969). [c] Underlined values are the same for isolated mycorrhizal and standard fatty acids. [d] Structure may be 10*c*-18:1.

values (Miwa et al., 1960) on the GLC phase SP-1000 revealed that four acids in the profile of lipids from seedlings grown in inoculated soil were different from the fatty acids normally found in higher plant material (Hitchcock, 1975) and from acids found in citrus juice and seeds (Nordby and Nagy, 1969) and leaves (Nordby et al., 1976).

Purification of the infected root FAME's by TLC with ethyl ether–hexane (1:9) as the solvent revealed that the four fatty acids stayed with the normal fatty acid esters, indicating these acids had no keto, epoxy, hydroxy, or dicarboxylic components. Hydrogenation of the total FAME's and subsequent GLC analyses gave 73% 16:0 and 2.2% 20:0 along with 3.0% iso-16, 1.7% anteiso-17, and 20.1% 18:0. These last three values correspond to the respective percentages of the two branched acids and the combined percentages of the four linear C-18 fatty acids in the samples before hydrogenation. The 73% represented both linear 16:0 and 16:1; and the 2.2% represented trace and measurable amounts of saturated and unsaturated C-20 acids, respectively. Since no other peaks were observed in the C-16 to C-20 area of the hydrogenated FAME chromatograms, the major three mycorrhizal fatty acids were determined to be linear C-16 and C-20's while the fourth (minor) acid appeared to be a linear C-18.

Preparative silver nitrate TLC of the FAME's along with standards revealed that of the mycorrhizal fatty acids, the C-16 acid was a monoene, the C-18 acid and one C-20 acid were trienes, and the other C-20 fatty acid was a tetraene. The monene fraction upon hydrogenation was shown by GLC to contain the expected 94% methyl palmitate and 6% methyl stearate. Similarly, when the two triene acids and the tetraene acid were hydrogenated and analyzed by GLC, they were converted to their respective 18:0 and 20:0 linear derivatives.

The ECL's of a number of standards and mycorrhizal fatty acids on four GLC liquid phases are given in Table I. Fatty acid 11c-16:1 had ECL values identical with those of synthetic 11c-16:1. Deviations in 11c-16:1's ECL values from those of standards palmitoleic (9c-16:1), 3-*trans*-hexadecenoic (3*t*-16:1), oleic (9c-18:1), and stearoleic acid methyl esters agree with deviations calculated for the 11c-16:1 structure (Marinari et al., 1974; Ackman and Castell, 1967; Ackman and Hooper, 1973a,b).

Further confirmation of this structure was obtained by mass spectra and ozonalysis studies. The MS of the C-16:1 pyrrolidide lost $-CH_2-$ ions consecutively, producing *m/e* 278, 264, 250, and 236 ions. The next ion at 224 *m/e* showed a 12-unit change instead of a 14-unit change. This was evidence that the acid was 11-16:1 (Andersson and Holman, 1973). Ozonolysis of the 16:1 methyl ester and subsequent GC–MS gave as the principal ion a C-11 aldehyde methyl ester with *m/e* 183 (M − 31), which confirms the 11th position of the double bond.

The three mycorrhizal polyenoic fatty acids had ECL values on three or four GLC phases identical with γ-linolenate (6*c*,9*c*,12*c*-18:3), 8*c*,11*c*,14*c*-20:3, and arachidonate (5*c*,8*c*,11*c*,14*c*-20:4) standards. In addition, the roots' 20:3 and 20:4 acid ECL's were very close to the calculated ECL values for the two respective structures calculated from ECL values of methyl linolenate (9*c*,12*c*,15*c*-18:3) obtained on the four liquid phases by the formula $ECL_x = aECL_{9,12,15-18:3} + b$, the values for the constants *a* and *b* being tabulated for each proposed polyene structure (Jamieson and Reid, 1969).

Mass spectral analyses and ozonlysis–MS analyses of the C-18:1 acid in the monoene fraction of infected roots revealed that at least the major portion of the C-18 monoenes had the double bond in the tenth position instead of the normal ninth (oleate) found in most organisms. Further studies are needed to confirm this finding that infected roots contain 10-18:1 and control roots 9-18:1.

Sterols and Fatty Acids of *G. mosseae* Chlamydospores. The major sterol (96%) had an RRT (retention time relative to that of cholesterol) on two GLC phases corresponding to campesterol (24α-methyl-Δ^5-cholestenol) (Table II). Campesterol is isomeric with 22-dihydrobrassicasterol (24β-methyl-Δ^5-cholesterol), the two sterols differing in their C-24 configuration. These two sterols are not resolvable by GLC or mass spectral analyses. In fungi ergosterol (24β-methyl-$\Delta^{5,7,12}$-cholestatrienol) and, to a lesser extent, fungisterol (24β-methyl-Δ^7-cholestenol) are the only sterols found at relative percentages greater than 90% (Weete, 1974). Ergosterol has been reported to have RRT's very near to those of campesterol (Knights, 1967). When an ergosterol standard was analyzed on our

Table II. Relative Percentages and GLC Relative Retention Times of Free Demethyl Sterols of *G. mosseae* Chlamydospores

sterol	%	rel retention time[a] Dexsil-300	SP-1000
cholesterol	0.9	1.00	1.00
ergosterol	–[b]	1.23	1.38
campesterol or 22-dihydrobrassicasterol	96.0	1.32	1.28
stigmasterol	0.3	1.41	1.36
fungisterol	–	1.54	1.50
β-sitosterol	2.8	1.64	1.52

[a] Relative to retention time of cholesterol: 566 s at 220 °C on Dexsil-300; 444 s at 205 °C on SP-1000. [b] Less than 0.1% if present.

Dexsil-300 and SP-1000 columns, resolution of ergosterol–campesterol was achieved. On both columns fungisterol, the Δ^7 isomer of campesterol, had RRT's 1.17 times the RRT of campesterol. This value is characteristic of Δ^7/Δ^5 RRT ratios (Knights, 1967; Itoh et al., 1974). Most fungal sterols have the C-24β configuration; thus the major sterol in *G. mosseae* chlamydospores is most likely 22-dihydrobrassicasterol.

In *G. mosseae* chlamydospores (experiment 2), triglycerides were the major components of the total lipid fraction, as obserbed by TLC (Table III). The fatty acid profiles of total lipids, triglycerides, and glyco-polar lipids were quite similar except in the concentration of a 20:5 fatty acid. This acid was present in low concentration in the glyco-polar lipids but made up over 14% of the triglycerides. Although restrictions on the amount of material prevented a definite structure determination, the ECL's of this acid on two of the GLC columns corresponded to calculated values for 5c,8c,11c,14c,17c-20:5 (Table I). The triglyceride FAME's after hydrogenation contained 14% 20:0 by GLC analyses. Fatty acids 20:3 and 20:4 were found in only trace amounts or were not detected in the spores.

Fatty Acid Profiles of Fibrous Roots Infected with Different *Glomus* Species. In general there was no distinct difference between the three *Glomus* species in their ability to transfer 11c,-16:1 to citrus seedlings, the 32–59.5% range in total lipid for this series of experiments agreeing with the 28–40% range found previously (Nagy et al., 1980) with *G. mosseae*. The age of the seedling appears to be a factor, however. Rough lemon seedlings were inoculated with *G. etunicatus* (experiment 5b) and with *G. mosseae* (Nagy et al., 1980) (experiment 5a) the seedlings being 133, 140, and 230 days old, repectively, when they were excised. Relative percentages of 11c,-16:1 in the total lipids of these infected roots were respectively 38.7, 39.5, and 54.4%. Specific rootstocks have been shown to respond in growth to different species of *Glomus* (Nemec, 1978). This seems to be the case in the transfer of 11c-16:1 with sour orange rootstock. With *G. fasciculatus* this rootstock contained 32% 11c-16:1 (Table III, experiment 3), while with *G. etunicatus* (Table III, experiment 4) this acid accounted for 59.5% of the fatty acids in the roots of sour orange seedlings.

Fatty Acids of Phosphate-Supplemented Citrus. In experiment 4 (Table III), nearly 60% of the fatty acids from roots grown in the inoculated soil was due to 11c-16:1, but this acid was not detected in the phosphate-supplemented plants. The two C-20 unsaturated fatty acids generally observed with 11c-16:1 likewise were present in the infected roots but not the high phosphate supplemented roots.

Location of Mycorrhizal Fatty Acids in Citrus Plant. In the leaves (experiment 5a) very little difference was observed between the C and M plants; the mycorrhizal fatty acids were not detected in the leaves. The C and M fatty acid profiles of the tap roots also were similar. The 2.1% 11c-16:1 in the M roots is thought to have been due

Table III. Relative Percent of C-16 to C-20 Fatty Acids in Control (C) and Mycorrhizal (M) Citrus and Chlamydospores

expt	fungus-rootstock(s)/lipid-tissue	Σ(16's + 17)[a] C	Σ(16's + 17)[a] M	Σ18's[b] C	Σ18's[b] M	11c-16:1 M	20:3 M	20:4 M	20:5 M
2	*G. mosseae*-chlamydospores								
	total		38.9		12.5	42.9	–[c]	–	5.7
	triglycerides		34.3		9.5	41.9	–	–	14.3
	glyco-polar lipids		43.6		15.8	40.4	–	–	0.2
3	*G. fasiculatus*-rangpur lime and sweet and sour oranges[d]								
	total fibrous (f.) roots	49.7	38.5	50.3	21.6	38.3[e]	0.8	0.8	–
	triglycerides f. roots	41.7	34.5	58.3	12.0	51.9[f]	0.8	0.8	–
	glyco-polar lipids f. roots	53.3	46.3	46.7	38.7	13.2	0.9	0.9	–
4	*G. etunicatus*-sour orange								
	total f. roots, high phosphate (C)	34.4		65.6					
	total f. roots (M)		20.4		15.8	59.5	1.5	2.8	–
5a	*G. mosseae*-rough lemon								
	total leaves	27.8	32.4	72.2	67.6	–	–	–	–
	total tap roots	24.6	27.0	75.4	70.9	2.1	–	–	–
	total f. roots	29.1	27.3	70.9	15.3	54.4	1.2	1.8	–
5b	*G. etunicatus*-rough lemon total f. roots	32.6	33.4	67.4	26.3	38.7	0.6	1.0	–
6a	*G. etunicatus* and *G. mosseae*-sour orange and rough lemon								
	phosphatidic acid	30.4	30.1	69.6	63.3	3.5	1.0	2.1	–
	phosphatidylinositol	51.4	45.3	48.6	46.3	3.4	2.1	2.9	–
	phosphatidylcholine	31.8	30.6	68.2	55.2	5.5	2.8	5.9	–
6b	*G. etunicatus*-rough lemon								
	esterified sterol glucoside	42.0	33.6	58.0	59.8	6.2	0.1	0.3	–
7	endomycorrhizal fungus not determined								
	total carrizo citrange (2 years) f. roots		26.6		43.6	27.3	0.9	1.6	–
	total grapefruit (9 years) f. roots		27.4		55.1	16.2	0.5	0.8	–

[a] Includes iso-16:0, 16:0, 9c-16:1, and anteiso-17:0. [b] Includes 18:0, 18:1, 18:2 and 18:3. [c] Less than 1% if present. [d] Mean values of the three rootstocks. [e] Values were as follows: rangpur lime, 48.8%; sweet orange, 34.1%; sour orange, 32.0%. [f] Values were as follows: rangpur lime, 57.7%; sweet orange, 47.6%; sour orange, 50.3%.

to contamination from fibrous roots since we had difficulty deciding on the line of demarcation between the fibrous roots and the tap roots.

Distribution of Mycorrhizal Fatty Acids in Various Lipids. The major concentration of the mycorrhizal fatty acids occurred in the triglycerides (Table III). As observed for *G. mosseae*, the glyco-polar lipid fraction from citrus roots inoculated with *G. fasciculatus* had a 15% content of the three major (16:1, 20:3, and 20:4) mycorrhizal fatty acids. When the glycolipid fraction was analyzed by TLC, the only major lipid to appear was ESG, the concentration being much heavier in the M samples. Each of the three major phospholipids had their own intrinsic profiles with very little differences observed between C and M profiles. The three major mycorrhizal fatty acids were individually not found above 6%, each phospholipid having different proportions of the three acids.

Mycorrhizal Fatty Acids in Older Citrus. For determination of whether there is a possibility of using the mycorrhizal fatty acids as a diagnostic tool for detecting mycorrhizal infection in citrus, two ages of citrus roots were examined for their fatty acid profiles (experiment 6b, Table III). Both roots showed the typical infected root fatty acid profile, with 11*c*-16:1, 20:3, and 20:4 present. The three major mycorrhizal fatty acids tended to decrease in percentages with the age of the tree. Since it has been shown that mycorrhizal infection is dependent on season, rootstock, and species of the fungus (Nemec, 1978), these three conditions will have to be known before the levels of these mycorrhizal fatty acids can be compared. The presence of these acids in older citrus trees indicates, however, that the lipid transfer mechanism of the mycorrhizae is not confined to young citrus seedlings but is still present in older trees.

DISCUSSION

As has been observed in previous studies, lipids are truly transferred from the fungus itself to the host citrus roots. Four fatty acids which are not generally found in higher plants were found in citrus roots infected with endomycorrhizae but not in roots of noninfected plants. This is the first report on the fatty acids associated with endomycorrhizal fungi. Fatty acids associated with ectomycorrhizal fungi have been shown to consist primarily of linoleic, palmitic, and oleic acids (Melhuish et al., 1975). Endomycorrhizal fungi belong in the family Endogonacea C order Endogonales of the Zygomycetes (Phycomycetes). These fungi of lower phylogenetic origin characteristically contain γ-linolenic (6,9,12-18:3) instead of linolenic acid (9,12,15-18:3), found in most fungi (Weete, 1974). Phycomycetes also contain greater percentages of C-20 to C-22 saturated and unsaturated fatty acids than the higher fungi (Weete, 1974). The presence of 6,9,12-18:3, 20:3, and 20:4 in the mycorrhizal roots of citrus clearly falls in line with these general chemotaxonomic observations for Phycomycetes.

Although 11*c*-16:1 has been reported to be a major component of seed oils (deTomas et al., 1963; Vickery, 1969), this is the first time, to the best of our knowledge, that it has been reported to be a major constituent of fungi. This acid is, however, a major constituent of a number of *Myxobacters* (Walker, 1969; Livermore et al., 1969; Noren and Odham, 1973).

The high proportion of 24-methylcholesterol in the free sterols of the *G. mosseae* chlamydospores is unusual for fungi (Weete, 1974). The data, however, complement previous findings that campesterol/total sterol ratios are greater for mycorrhizal than for noninfected roots (Ho, 1977; Nagy et al., 1980).

The fatty acid composition of the fungal spores are generally qualitatively similar to that of the parent mycelia (Weete, 1974). Thus, a comparison of the corresponding fatty acid profiles of the various lipid fractions from those of the spores and roots suggests that intact triglycerides are transfered more completely than other lipid components from the fungus to the roots.

The fatty acids of the fungal glyco-polar lipid components consisted of over 40% 11*c*-16:1. In the infected roots, however, less than 14% of the glyco-polar lipid fatty acids and less than 6% of the fatty acids in each of the three major phospholipids consisted of this mycorrhizal fatty acid. This appears to rule out the possibility that the phosphate is transferred from the fungus to the host as a phospholipid. Further evidence against this possibility is the lack of increased lipids and specifically 11*c*-16:1 fatty acid in noninoculated citrus grown on high phosphate.

The lack of the mycorrhizal fatty acids in portions of the infected citrus plant other than the fibrous roots is evidence that for citrus, lipids may have a passive role in increasing the growth of mycorrhizal plants. They may, for example, serve as a pool or storehouse of energy in the roots of the plant. A search of specific portions of the root–fungus system such as vesicles, arbuscules, and intercellular areas, as well as external hyphae regions, of the fibrous root for these specific mycorrhizal fatty acids would be of great interest.

ACKNOWLEDGMENT

We thank Wayne Heimermann, Hormel Institute, University of Minnestoa, Austin, MN, for the MS analyses and Dr. H. Goldfine, University of Pennsylvania, for a sample of synthetic 11*c*-16:1 fatty acid.

LITERATURE CITED

Ackman, R. G.; Castell, J. D. *J. Gas Chromatogr.* **1967**, *5*, 489.
Ackman, R. G.; Hooper, S. N. *J. Chromatogr.* **1973a**, *86*, 73.
Ackman, R. G.; Hooper, S. N. *J. Chromatogr.* **1973b**, *86*, 83.
Andersson, H. A.; Holman, R. T. *Lipids* **1973**, *9*, 185.
Cooper, K. M.; Losel, D. M. *New Phytol.* **1978**, *80*, 143.
Cox, G.; Sanders, F. *New Phytol.* **1974**, *73*, 901.
Cox, G.; Sanders, F.; Tinker, P. B.; Wild, J. A. In "Endomycorrhizas"; Sanders, R. E.; Mosse, B.; Tinker, P. B., Eds.; Academic Press: London, 1975; pp 297–306.
deTomas, M. E.; Brenner, R.; Cattaneo, P. *Rev. Argent. Grasas Aceites* **1963**, *5*, 53; *Chem. Abstr.* **1964**, *61*, 3314*g*.
Dittmer, J. C.; Lester, R. L. *J. Lipid Res.* **1964**, *5*, 126.
Gerdemann, J. W. *Annu. Rev. Phytopathol.* **1968**, *6*, 397.
Harley, J. L. In "Endomycorrhizas"; Sanders, R. E.; Mossee, B.; Tinker, P. B., Eds.; Academic Press: London, 1975; pp 1–24.
Hitchcock, C. In "Recent Advances in the Chemistry and Biochemistry of Plant Lipids"; Galliard, T.; Mercer, E. I., Eds.; Academic Press: New York, 1975; pp 1–20.
Ho, I. *Lloydia* **1977**, *40*, 476.
Itoh, T.; Tamura, T.; Matsumoto, T. *Lipids* **1974**, *9*, 173.
Jamieson, G. R.; Reid, E. H. *J. Chromatogr.* **1969**, *42*, 304.
Kleinschmidt, G. D.; Gerdemann, J. W. *Phytopathology* **1972**, *62*, 1447.
Knights, B. A. *Mem. Soc. Endocrinol.* **1967**, *16*, 211.
Livermore, B. P.; Johnson, R. C.; Jenkin, H. M. *Lipids* **1969**, *4*, 166.
Losel, D. M.; Cooper, K. M. *New Phytol.* **1979**, *83*, 415.
Marinari, L. A.; Goldfine, H.; Panos, C. *Biochemistry* **1974**, *13*, 1978.
Marx, D. H.; Bryan, W. C.; Campbell, W. A. *Mycologia* **1971**, *63*, 1222.
Melhuish, J. H.; Hacskaylo, E.; Bean, G. A. *Mycologia* **1975**, *67*, 952.
Miwa, T.; Mikolajczak, K. L.; Earle, F. R.; Wolff, I. A. *Anal. Chem.* **1960**, *32*, 1739.
Nagy, S.; Nordby, H. E.; Nemec, S. *New Phytol.* **1980**, *85*, 377.
Nemec, S. *Proc. Fla. State Hortic. Soc.* **1978**, *91*, 10.
Nordby, H. E.; Nagy, S. *Phytochemistry* **1969**, *8*, 2027.

Nordby, H. E.; Nagy, S.; Hall, N. *Phytochemistry* **1976**, *15*, 957.
Noren, B.; Odham, G. *Lipids* **1973**, *8*, 573.
Peterson, J. I.; Schmertzing, H.; deAbel, K. *J. Gas Chromatogr.* **1965**, *3*, 126.
Vickery, J. R. *Aust. J. Sci.* **1969**, *31*, 334.
Walker, R. W. *Lipids* **1969**, *4*, 15.
Weete, J. D. In "Fungal Lipid Biochemistry"; Weete, J. D., Ed.; Plenum Press: New York, 1974; pp 74–79, 92, 158–161.

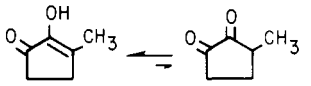
Received for review April 7, 1980. Accepted November 4,1980. Mention of a trademark or proprietary product is for identification only and does not recommend approval by the U.S. Department of Agriculture over other products that may also be suitable.

Quantitative Analysis of Alkyl-2-hydroxy-2-cyclopenten-1-ones in Tobacco Smoke

Stephen S. Hecht,* Steven Carmella, and Dietrich Hoffmann

2-Hydroxy-3-methyl-2-cyclopenten-1-one-*3-methyl*-^{14}C (1-*3-methyl*-^{14}C) was synthesized and employed as an internal standard for the quantitative analysis of 1 and related alkyl-2-hydroxy-2-cyclopenten-1-ones in cigarette smoke. The mainstream smoke of a typical U.S. blended 85-mm nonfilter cigarette contained 52 ± 3 μg of 1 as well as 3,5-dimethyl-2-hydroxy-2-cyclopenten-1-one (9.9 μg), 3,4-dimethyl-2-hydroxy-2-cyclopenten-1-one (2.1 μg), and 3-ethyl-2-hydroxy-2-cyclopenten-1-one (24 μg). The level of 1 in cigarette smoke was selectively reduced by cellulose acetate filter tips. The smoke of cigarettes made from Bright tobacco contained more 1 than observed in the smoke of Burley or Oriental cigarettes. Levels of 1 in smoke were reduced by extraction of tobacco with hexane–ethanol or by use of tobacco from the bottom stalk position. A correlation between concentrations of 1 and catechol in cigarette smoke was observed, indicating common leaf precursors for these two components.

Alkyl-2-hydroxy-2-cyclopenten-1-ones have been detected in tobacco smoke by several groups (Elmenhorst, 1972; Morêe-Testa and De Salles de Hys, 1975; Hecht et al., 1975; Schumacher et al., 1977; Sakuma and Sugawara, 1979). 2-Hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene, 1) is the most abundant compound in this group. It



1

exists predominantly as the enolic tautomer and is therefore detected in the weakly acidic or phenolic fractions of tobacco smoke (Son Bredenberg, 1959). We initially became interested in 1 because of its presence in subfractions of the weakly acidic fraction of cigarette smoke condensate which showed cocarcinogenic and tumor-promoting activity on mouse skin (Hecht et al., 1981). However, subsequent assays of 1 demonstrated that it was not active as a cocarcinogen or tumor promoter. The flavor properties of tobacco smoke, as well as numerous foods, are also influenced by 1, which has been described as having a sweet aroma and a sweet or maple taste (Elmenhorst, 1972; Leffingwell, 1972). Thus, the levels of 1 and related compounds in various products are important in determining their organoleptic properties. In the present study, we have developed a method for the quantitative analysis of 1 employing ^{14}C-labeled 1 as an internal standard.

EXPERIMENTAL SECTION

Reagents. All solvents were spectroquality. Ethyl 2-oxocyclopentanecarboxylate was obtained from Aldrich Chemical Co., Milwaukee, WI. $^{14}CH_3I$, 31.0 mCi/mmol, was procured from New England Nuclear, Boston, MA. TLC was performed with Merck precoated silica gel 60 F-254 plates.

Apparatus. Cigarettes were smoked on a Heinr. Borgwaldt Automatic Smoking Machine RM-20/68. GLC was performed with a Hewlett-Packard Model 5830A gas chromatograph equipped with a Model 18835B capillary inlet system, a flame ionization detector, and columns A [30 m × 0.25 mm Carbowax 20M (Supelco, Inc, Bellefonte, PA)] and B [6 ft × 2 mm glass column packed with 10% XE-60 on Chromosorb WHP]. GLC–MS was carried out with a Hewlett-Packard Model 5982A instrument. High-performance LC was done with a Waters Associates Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 660 solvent programmer, a Model U6K septumless injector, a Model 440 UV-visible detector, and a 3.9 mm × 30 cm μBondapak C_{18} column (Waters Associates, Milford, MA). Thin-layer radiochromatography was performed with a Packard Model 7201 radiochromatogram scanner. Scintillation counting was done with a Nuclear Chicago Isocap 300 system. UV spectra were run on a Cary Model 118 spectrometer.

Cigarettes. Cigarettes A–D were obtained on the open market in 1979. The Burley cigarettes of differing stalk positions were obtained through the courtesy of Dr. T. C. Tso, Tobacco Laboratory, U.S. Department of Agriculture, Beltsville, MD. The hexane–ethanol azeotrope extracted cigarettes (Gori, 1980), the corresponding unextracted cigarettes (standard experimental blend IV), and the modified cellulose cigarettes were made available by Enviro-Control, Rockville, MD.

2-Hydroxy-3-methyl-2-cyclopenten-1-one-*3-methyl*-^{14}C. A mixture of potassium ethyl 2-oxocyclopentanecarboxylate (3) (Mayer et al., 1958) (770 mg, 3.97 mmol) and $^{14}CH_3I$ (55.2 mg, 0.4 mmol, 5.0 mCi/mmol) in 1.5 mL of dry Me_2SO was stirred for 40 h at room tem-

Divisions of Chemical Carcinogenesis and Environmental Carcinogenesis, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595.

0021-8561/81/1429-0401$01.25/0