THE OXYGENATED FATTY ACIDS FROM THE OIL OF THE SPORES OF LYCOPODIUM SPECIES[1,2]

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ABSTRACT

The oil from the spores of commercial *Lycopodium*, mainly *L. complanatum*, contains *cis*-9,10-epoxyoctadecanoic acid (2%), (+)-8-hydroxyhexadecanoic acid (7%), and (±)-*threo*-9,10-dihydroxyoctadecanoic acid (4%). Smaller amounts of 8-oxohexadecanoic acid and an epoxyhexadecanoic acid also seem to be present.

The oil from the spores of species of the clubmoss *Lycopodium* has been examined by a number of workers, most of whom (1–5) agreed that it contained a small amount of a 9,10-dihydroxyoctadecanoic acid. The present investigation of the spore oil was undertaken because it was thought that the dihydroxy acid might have been produced in the spore by enzymatic hydrolysis of a hitherto unobserved epoxyoctadecanoic acid. The enzymatic conversion of *cis*-9,10-epoxyoctadecanoic acid to (+)-*threo*-9,10-dihydroxyoctadecanoic acid had earlier been found to occur in the uredospores of the wheat stem rust fungus (6). It was hoped that *Lycopodium* spores might prove to be a more convenient source of epoxide-hydrolyzing enzyme than wheat stem rust spores.

A further reason for reinvestigating *Lycopodium* spore oil was that the early workers disagreed about the melting point of the dihydroxy acid. Thus Bukowsky (1) gave 92 °C, Rathje (2) 140 °C, Bauer and Piners (3) 98 °C, Riebsomer and Johnson (4) 93 °C, and Hirai and Toyama (5) 130 °C. These melting points indicate that different stereoisomers had been isolated; the higher melting acids probably were *erythro* forms (racemic form has melting point 130 °C and the (+) or (−) form 140 °C) and the lower melting probably *threo* forms (racemic form has melting point 95 °C and the (+) or (−) form 99 °C). One possible explanation for these results is that the spores had been obtained from different species of *Lycopodium*. The above workers seem to have used commercial "lycopodium powder", and though it has been assumed that this consists of the spores of *L. clavatum*, it is most probable that other species are also present in considerable proportions. The spores of the "lycopodium powder" used in the present work belonged to the species *L. complanatum*.

Methyl esters of the oil were prepared, and analyzed by gas–liquid chromatography (g.l.c.) using a silicone SE-30 column. The g.l.c. separation of the esters is shown in Fig. 1A and the separation, after the esters had been treated with acetic acid to open epoxide rings and acetylated to convert hydroxy esters to acetates (6, 7), is shown in Fig. 1B. The small peak in the C_{20} region in A is still smaller in B, indicating the presence of C_{18} epoxy ester (7). A diacetate peak has appeared in B, part of which should be derived from epoxy ester and the rest from dihydroxy ester in the original oil. Also in B an acetoxy palmitate peak has appeared just beyond the C_{18} peak. In A the original hydroxy palmitate would be obscured by the larger C_{18} peak, since previous work has shown that the

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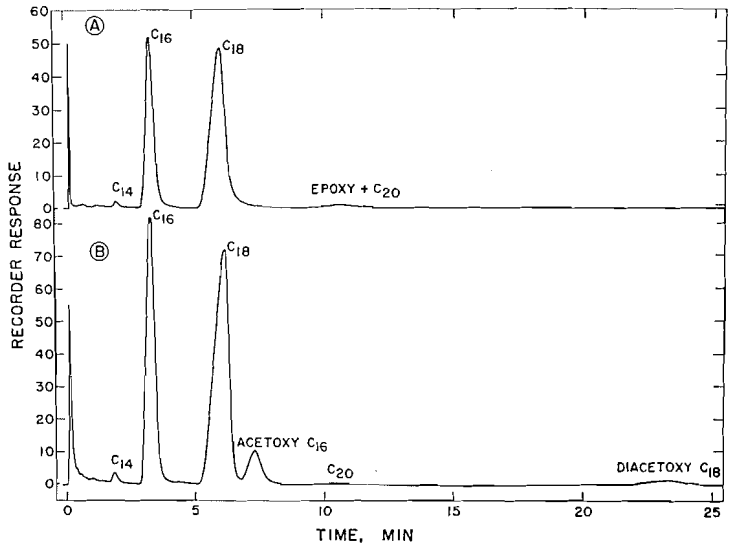

FIG. 1. (A) Gas–liquid chromatographic separation of the methyl esters of *Lycopodium* spore oil; (B) g.l.c. separation of esters after treatment with acetic acid and acetylation.

carbon number (8) of a hydroxy ester changes by 0.5 to 1.0 units on acetylation, the size of the change depending on the location of the hydroxyl group on the fatty acid chain (9).

Using a silicic acid column the esters were separated into non-oxygenated, epoxy, hydroxy, and dihydroxy ester fractions (10). The non-oxygenated esters were examined by g.l.c. and separated into saturated monoene and diene fractions by chromatography on a silver nitrate – silicic acid column (11, 12) and the fractions oxidized to determine the positions of the double bonds. 9-Hexadecenoic acid was present in the unusually large amount of 29%, as found by Riebsomer and Johnson (4), the other acids were oleic (21%) and linoleic (19%). The less common 11-octadecenoic acid, the presence of which was suggested by Riebsomer and Johnson, was also found (8%). The infrared spectra indicated that these unsaturated acids had the *cis* configuration.

Saponification of the epoxy ester fraction yielded an epoxy acid identical with the optically active *cis*-9,10-epoxyoctadecanoic acid previously isolated from the oil of wheat stem rust uredospores (13). Since the specific rotation of the epoxy ester was lower than that of epoxy ester derived from rust spores some racemic ester may also have been present in *Lycopodium* spore oil. The epoxy ester fraction also contained a lesser amount of a slightly more polar second component which was isolated after conversion of the accompanying epoxy ester to dihydroxy acid. The infrared spectrum indicated that it was an oxo ester (14); the carbon number, using a silicone QF-1 column, was 21.03 which suggested an 8- or 9-oxohexadecanoate (9). The acid could not be isolated in a sufficiently pure form for comparison with synthetic 8-oxohexadecanoic acid, but chromic acid oxidation yielded suberic and pimelic acids, confirming that it was 8-oxohexadecanoic acid. The fraction also contained a very small amount of a methyl epoxyhexadecanoate which had about the same carbon number, using the SE-30 column, as the above oxo ester but, using the QF-1 column, the carbon number was 20.2 (methyl *cis*-9,10-epoxyoctadecanoate had a carbon number of 22.2 using this column). This component could not be detected after acetic acid treatment (7).

Gas–liquid chromatography showed that the hydroxypalmitate fraction contained only

a single component. The ester was optically active and chromic acid oxidation at 100 °C yielded suberic and pimelic acids, indicating that the hydroxyl group was at carbon 8. Chromic acid oxidation at 25 °C gave an oxo ester which was shown to be methyl 8-oxopalmitate by comparison with the synthetic ester prepared by chain elongation of 6-oxotetradecanoic acid (9). The hydroxy ester is thus (+)-methyl 8-hydroxyhexadecanoate; (±)-methyl 8-hydroxyhexadecanoate was synthesized for comparison by reduction of the oxo ester. This hydroxy acid does not seem to have been isolated previously. The (±) ester has been synthesized before (15), but the route used and the physical properties of the ester and acid have apparently not been disclosed. The other hydroxyhexadecanoic acids which have been found in nature are 3-D-hydroxyhexadecanoic in the extracellular glycolipids produced by species of *Rhodotorula* (16), (+)-11-hydroxyhexadecanoic in the seeds and roots of convolvulaceous plants (17), 14- and (or) 15-hydroxyhexadecanoic in beeswax (18, 19), and 15- and 16-hydroxyhexadecanoic acids in the glycolipid produced by *Torulopsis magnoliae* (20).

The dihydroxy ester fraction was shown by comparison with synthetic material to be racemic methyl *threo*-9,10-dihydroxyoctadecanoate. Since the residue obtained after crystallization of the racemic *threo* ester had a low rotation ($[\alpha]_D$ +4°), the amount of (+)-*threo*-dihydroxy acid isomer ($[\alpha]_D$ +24° (13)) present in the oil, if any, must have been very small. The finding of a *threo* dihydroxy acid is in agreement with the results of Riebsomer and Johnson (4), who carried out the most thorough of the previous investigations.

The overall percentage fatty acid composition of the oil was calculated to be: myristic, 1; palmitic, 6; 9-hexadecenoic, 29; stearic, 1; 9-octadecenoic, 21; 11-octadecenoic, 8; linoleic, 19; *cis*-9,10-epoxyoctadecanoic, 2; 8-oxohexadecanoic, 0.4; (+)-8-hydroxyhexadecanoic, 7; *threo*-9,10-dihydroxyoctadecanoic, 4; and unidentified, 1.6.

The presence of racemic dihydroxy acid is surprising, particularly since the other two oxygenated acids are optically active. This acid could not have been produced by enzymatic hydrolysis of the epoxy acid as occurs in wheat stem rust spores unless it is assumed that some type of racemizing enzyme is also present in *Lycopodium* spores. The dihydroxy acid could not have arisen by hydrolysis of epoxy acid during preparation of methyl esters from the oil since it was previously shown that methanolysis with a weak solution of sodium methoxide in methanol did not affect the epoxide group (7, 26). It is unlikely that the oxygenated acids are produced by autoxidation since some are optically active and since only a few specific acids are produced. If random autoxidation occurred epoxy- and dihydroxy-hexadecanoic acids derived from 9-hexadecenoic acid would be expected.

It is interesting that about 2% of racemic *threo*-9,10-dihydroxyoctadecanoic acid was recently found in the oil of *Onguekoa gore* (isano oil) (21). This oil was also reported to contain 1.4% of *cis*-9,10-epoxyoctadecanoic acid, apparently mainly as the racemic form (22). The epoxy acid also occurs in small quantity in the seed oil of *Tragopogon porrifolius* (23). However, no explanation of the presence of these small amounts of oxygenated acid has been put forward. Epoxy and hydroxy acids have also been found in sulfur olive oil and it has been suggested that they are produced by the action of microorganisms (24, 25). At least some of the oxygenated acids in *Lycopodium* spore oil and the other oils may have been produced in the same way.

EXPERIMENTAL

Optical rotations were measured at 25 °C in a 1 dm tube. Light petroleum refers to a fraction of boiling range 60–80 °C.

Isolation of the Oil

The *Lycopodium* spores were purchased from Canadian Laboratory Supplies, Ltd., Winnipeg, and were found to consist mainly of spores of *Lycopodium complanatum* by Dr. M. Steeves of the Department of Geology, University of Saskatchewan. The spores were extracted by grinding in a ball mill with light petroleum for 48 h, the yield of oil was about 50%. The oil was treated with diazomethane to esterify free fatty acids, and, using methyl eicosanoate as a marker, the free fatty acids were estimated by g.l.c. to be less than 0.5%. Methyl esters were prepared by methanolysis with 0.02 *N* sodium methoxide in methanol (26)

Gas–liquid Chromatographic Analysis

Silicone SE-30 and QF-1 columns were prepared and operated as described earlier (9), and the ethylene glycol phthalate column used to analyze the esters and their oxidation products was employed as described by Tulloch and Craig (27).

Column Fractionation of the Methyl Esters of the Oil

Methyl esters (24.8 g) were dissolved in light petroleum and applied to a column (5 × 40 cm) of silica gel (Davison, 60–200 mesh). Non-oxygenated esters (20.5 g) were eluted with light petroleum containing 3% ether, and an epoxy ester fraction (1.1 g) with light petroleum containing 4% acetone; increasing the acetone content to 8% eluted a hydroxy ester fraction (1.5 g), and to 15% gave a dihydroxy ester fraction (1.1 g).

The Non-Oxygenated Ester Fraction

The following composition (in weight %) was obtained by g.l.c.: 14:0, 0.8; 14:1, 0.2; 16:0, 7.5; 16:1, 31.0; 16:2, 0.4; 18:0, 0.6; 18:1, 34.4; 18:2, 23.4; 20:0, 0.4; 20:1, 0.3; 22:0, 0.4; 22:1, 0.3. Chromatography on a silver nitrate – silicic acid column (11, 12) gave saturated esters (9.0), monoenes (65.9), and dienes (25.1%). Gas–liquid chromatography showed that the diene fraction contained some 16 carbon monoene but rechromatography on a shorter column yielded an almost pure diene fraction. The monoene fraction was separated, in batches, into C_{16} and C_{18} monoenes by g.l.c., using the SE-30 column. A portion of the C_{16} monoene was converted to *threo*-9,10-dihydroxyhexadecanoic acid by performic acid oxidation (26); the acid had melting point and mixed melting point 88–89 °C. The two monoene fractions and the diene fraction were oxidized with the permanganate periodate reagent (28) and the oxidation fragments analyzed by g.l.c. (27). The results are summarized below, only the major products are shown.

	C_{16} monoene (mole %)	C_{18} monoene (mole %)	Diene (mole %)
Dibasic acids			
C_9	94.2	69.4	93.6
C_{11}	1.2	26.9	1.3
Monobasic acids			
C_6	1.6	—	89.5
C_7	95.9	27.7	6.2
C_9	1.2	72.3	—

Epoxy Ester Fraction

Ester (0.90 g) was rechromatographed on a short silicic acid column and elution with light petroleum containing 6% ether gave almost pure epoxy ester (0.54 g). After crystallization from light petroleum the ester had melting point 22.5–24.5 °C and $[\alpha]_D$ +0.4° (*c*, 12.5, MeOH). A freshly prepared sample of methyl *cis*-9,10-epoxyoctadecanoate from the oil of wheat stem rust uredospores had melting point 23–25 °C and $[\alpha]_D$ +0.7° (*c*, 17.5, MeOH). The *Lycopodium* epoxy ester was saponified and the acid obtained after acidification was crystallized from acetone. The melting point and mixed melting point with *cis*-9,10-epoxyoctadecanoic acid from wheat stem rust was 57.5–58.5 °C and the mixed melting point with synthetic racemic *cis*-9,10-epoxyoctadecanoic acid was 53–57 °C. The infrared spectra of the acid from *Lycopodium* spores and the acid from wheat stem rust spores were indistinguishable.

A later fraction (0.21 g), obtained during the rechromatography of the epoxy ester, which seemed from g.l.c. analysis to be a mixture of an oxo C_{16} ester (40%) and the epoxy ester, was refluxed with acetic acid, the product hydrolyzed, and the acids isolated. The oxo acid was extracted with light petroleum and had melting point 72–77 °C, oxidation with chromic acid at 100° gave pimelic and suberic acids. The dihydroxy acid produced from the epoxy acid was crystallized from ethyl acetate and the melting point and mixed melting point with synthetic racemic *threo*-9,10-dihydroxyoctadecanoic acid was 93–94.5 °C.

Hydroxy Ester Fraction

Saponification of the ester yielded (+)-8-hydroxyhexadecanoic acid which, after crystallization from acetone, had melting point 78.5–79.5 °C and $[\alpha]_D$ +0.3° (*c*, 19.5, $CHCl_3$).

Anal. Calcd. for $C_{16}H_{32}O_3$: C, 70.5; H, 11.8. Found: C, 70.5; H, 11.9.

Methyl (+)-8-hydroxyhexadecanoate was prepared by treating the acid with diazomethane. Crystallization from light petroleum gave the pure ester with melting point 44–45.5 °C and $[\alpha]_D$ +0.8° (*c*, 14, MeOH).

Anal. Calcd. for $C_{17}H_{34}O_3$: C, 71.3; H, 12.0. Found: C, 71.4; H, 12.1.

The mixed melting point with the racemic ester was 42.5–45 °C.

Methyl ester (0.01 g) was oxidized with a solution of chromic acid in glacial acetic acid (2%, 1 ml) for 30 min at 100°. Sodium metabisulfite and dilute sulfuric acid were added and the products extracted with ether and converted to methyl esters with diazomethane. Gas–liquid chromatographic analysis on the polyester column showed that the major dibasic products were pimelic and suberic acids.

Methyl 8-oxohexadecanoate was prepared by oxidation of the hydroxy ester with chromic acid in acetic acid at 25 °C. The melting point of the oxo ester and the mixed melting point with synthetic methyl 8-oxohexadecanoate was 36–36.5 °C. 8-Oxohexadecanoic acid was obtained on hydrolysis and had melting point and mixed melting point 75.5–77 °C.

Synthesis of 8-Oxo- and 8-Hydroxyhexadecanoic Acids

6-Oxotetradecanoic acid (6 g) (9) and methyl hydrogen succinate (6.6 g) were electrolyzed in 0.022 *N* sodium methoxide solution (30 ml). After 1.5 h a further 3 g of half ester was added in sodium methoxide solution (10 ml) and the electrolysis continued for another 1.5 h when the solution was alkaline. Methanol was added to give a volume of 75 ml and insoluble dioxohexacosane was filtered off. The residue was acidified with acetic acid, the methanol removed, and the product taken up in ether and washed with sodium bicarbonate. After removal of the ether the esters were distilled and crude oxo ester collected at 130–150 °C and 0.1 mm. The distillate was saponified and crystallization of the acid from acetone yielded 8-oxohexadecanoic acid (1.35 g) with melting point 76–77 °C (lit. (29) gives 77.4–78.9 °C). The methyl ester had melting point 36–36.5 °C (lit. (29) gives 36.5–37.1 °C).

Hydrogenation of the oxo acid (30) yielded 8-hydroxyhexadecanoic acid with melting point 73.5–74.5 °C.

Anal. Calcd. for $C_{16}H_{32}O_3$: C, 70.5; H, 11.8. Found: C, 70.5; H, 12.0.

The methyl ester had melting point 45–46 °C.

Anal. Calcd. for $C_{17}H_{34}O_3$: C, 71.3; H, 12.0. Found: C, 71.5; H, 12.0.

Dihydroxy Ester Fraction

Crystallization from light petroleum gave methyl *threo*-9,10-dihydroxyoctadecanoate with melting point 67.5–68.5 °C, undepressed by admixture with the synthetic ester of melting point 68–69 °C. After removal of several optically inactive crops of crystals from the mother liquors the residue (0.17 g from 1.16 g) had $[\alpha]_D$ +4° (*c*, 3.4, MeOH). *threo*-9,10-Dihydroxyoctadecanoic acid was obtained by saponification of the ester and had melting point and mixed melting point of 93–94.5 °C.

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