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A Prostaglandin-Like Fatty Acid from a Species in the Cyperaceae

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ABSTRACT

A C_{20} cyclic trihydroxy unsaturated fatty acid was isolated and characterized as a representative member of a group of oxygenated fatty acids from the aquatic sedge, *Eleocharis microcarpa,* Torr. Characterization of the compound as 11-hydroxy-14-(3,5-dihydroxy-2-methylcyclopentyl)-tetradec-9-ene-12-yneoic acid was accomplished by various chemical and spectral methods.

Lipids 18:617-622, 1983.

INTRODUCTION

In the course of our investigation of the allelopathic relationship between the fresh water sedge, *Eleocharis microcarpa*, Torr., and blue-green algae (Cyanochloronta), a relatively large number of hydroxy and hydroxy keto free fatty acids were isolated. These substances appear to occur naturally in *E. microcarpa* and are implicated as the allelopathic agents (1).

One of the compounds was investigated more thoroughly than the others and was characterized as 11-hydroxy-14-(3,5-dihydroxy-2-methylcyclopentyl)-tetradec-9-ene-12-yneoice acid. In view of its similarity to prostaglandins,e to prostaglandin-like compounds producede enzymatically from other plants (2), and thee importance of lipid peroxidation in general (3,e 4), the occurrence should be of interest toe other investigators. This class of compoundse may have significance to algal succession (5).e

MATERIALS AND METHODS

Plant Material

E. microcarpa, was collected from pondse located near Hattiesburg, Mississippi, and was extracted fresh or fresh frozen. A voucher specimen is on file in the University of Southern Mississippi herbarium.

Bioassays

Bioassays were used to follow activity against blue-green algae through each step of isolation and final purification. It was found that using sensitivity disks on agar plates sprayed with the challenge organism *Anabaena flos-aqua* gave results within 36 hr. The diameter of the zone of inhibition gave a rough measure of specific activity.

Chemicals

All solvents used in this investigation, except for high performance liquid chromatography (HPLC), were purchased as reagent grade and distilled in glass prior to use. HPLC grade was used for retention time studies. All solvent mixture ratios were v/v. Trimethylsilane derivatives were prepared with DMB Sil Prep (Applied Science Laboratories, State College, PA). Diazomethane used in making methyl esters was generated from Diazald, N-methyl-N-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Company, Milwaukee, WI). 2-Thiobarbituric acid (TBA) and prostaglandins PGA_1 and PGE_1 were purchased from Sigma Chemical Company (St. Louis, MO).

Extraction

Wet *E. microcarpa* (typically 2 kg) was refluxed in 2 l of distilled water for ca. 1 hr and then allowed to cool. After Filtering, the resulting liquid was acidified to a pH of 2 with conc HCL and extracted 3 times with 200-ml portions of chloroform. The chloroform layers were combined and dried with anhydrous sodium sulfate. The chloroform was removed under reduced pressure. This procedure yielded 0.2 g of waxy yellow semisolid material.

Column Chromatography

Two cm glass columns of 80-200 mesh silica gel (50 g) were used. A 0.5-g portion of the crude extract was eluted with 300 ml of each chloroform (fraction I), 1:1 chloroform/acetone (fraction II), acetone (fraction III) and methanol (fraction IV).

Analytical and Preparative Thin Layer Chromatography (TLC)

Further purification of fraction II by TLC was difficult because of the large number of compounds and their functional similarity. Four solvent systems were employed for both

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analytical and preparative TLC: system I, 85:15:2, chloroform/methanol/water; system II, 20:1, chloroform/methanol; system III, 60:40:2, hexane/ethyl ether/acetic acid; and, system IV, 50:50:2, hexane/ethyl ether/formic acid. Analytical TLC was performed on precoated sheets of Silica Gel 60, F-254, 0.2 mm, on aluminum backing purchased from Brinkmann Instruments, Inc. (Westbury, NY).

Preparative TLC was done in 2 stages: first, solvent system I was used; second, after isolation, each band was chromatographed again using system IV. Separation procedures are summarized in Figure 1. The scheme was developed after much trial and error and the use of bioassay results for guidance. For simplicity, only R_f values for bands separated by rechromatography of band 4 are shown. All preparative plates were made in these laboratories using Brinkmann Silica Gel 60 with F-254 indicator at a thickness of 0.5 mm on 20 x 20 cm glass plates. From 15 to 20 mg of material to be separated were applied to each plate. Bands were visualized under UV light, scraped from the plates and eluted with 2:1 chloroform/methanol. All chemical and spectral studies were performed on freshly purified material.

Analytical HPLC

Retention times of components in fraction II were compared with prostaglandins PGA_1 and PGE_1. The comparison was made on an Alltech C_{18} column, 25 cm x 4.6 mm, 5 μ using a linear gradient of H_2O/methanol starting with 2:1 v/v and ending with 100% methanol. The pH of the water component was adjusted to 3.6 with acetic acid to retard ionization of the free acids. A Laboratory Data Control UV detector set at 280 nm was used to monitor eluted components.

Derivatization and Tests

Methyl esters were prepared with diazomethane, using methods described in the literature. Trimethylsilyl ether and ester derivatives were prepared by reacting ca. 1 mg of sample with 1 ml of DMF Sil Prep under anhydrous conditions for a minimum of 12 hr. Shorter time periods did not produce complete silylation. Ozonolysis was accomplished by passing ozone through 1-mg samples dissolved in chloroform followed by reduction with 5 mg of triphenylphosphine at room temperature overnight. Procedures for the TBA test (5) and for ketones using 2,4-dinitrophenylhydrazine are

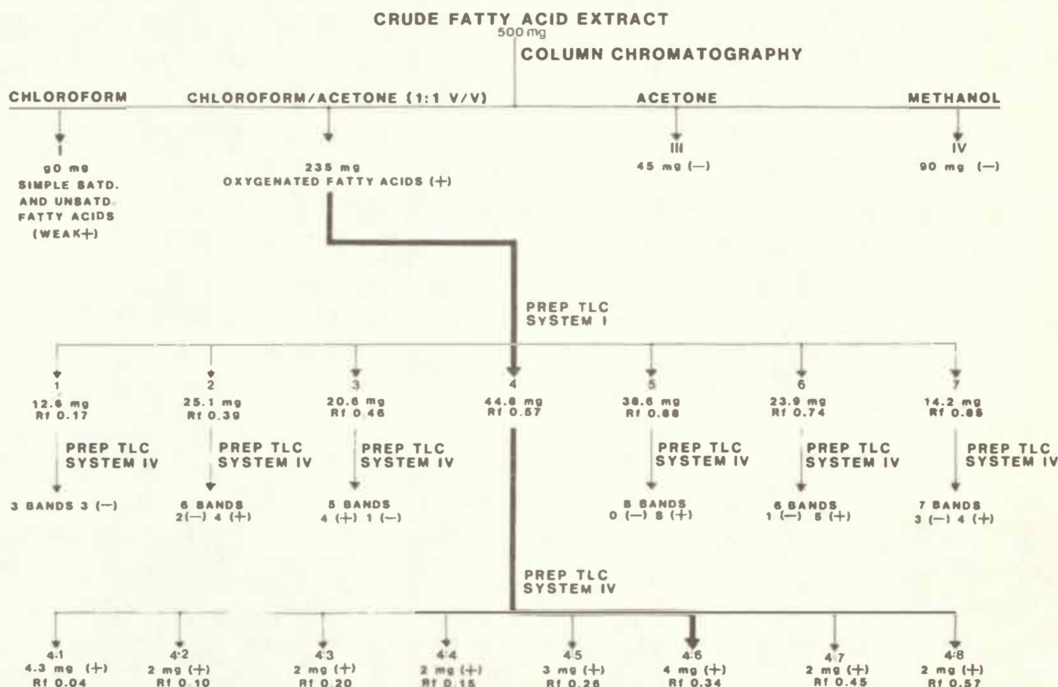

FIG. 1. Column and preparative TLC. Bioassays are shown in parentheses. (+) denotes at least a minimum detectable zone of inhibition around paper disc.

described in the literature.

Spectral Analysis

Infrared spectra were taken next on a Perkin Elmer 567 spectrophotomer and ultraviolet spectra with a Varian Cary 17 spectrophotometer. Low resolution mass spectra (MS) were obtained with a Dupont 2149 mass spectrometer. When gas chromatography (GLC) effluents of silylated derivatives were scanned, this instrument was connected to a Varian 2740 equipped with 6 ft × ⅛ in. stainless steel column packed with 3% Dexsil 300 on Chromosorb W AW. High resolution spectra were done at the Florida State University High Resolution Mass Spectroscopy Laboratory on an AEI MS-9. Both spectrometers were operated at an electron potential of 70 eV. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a JEOL FX90Q Fourier transform spectrometer.

RESULTS AND DISCUSSION

We chose to examine the oxygenated fatty acids in *E. microcarpa* because of bioassay data and previous reports (6,7) that suggested these materials may be natural algal inhibitors. Extraction of plant material with boiling water and partitioning into chloroform resulted in reasonable yields of free fatty acids that were relatively free of pigments and of other substances tending to interfere with column and TLC.

Fraction II, from column chromatography, gave typical carboxylic IR absorption (8) between 2850 cm^{-1} and 2350 cm^{-1} and at 1710 cm^{-1}. A positive test with 2,4-DNP and a strong IR absorption at 3400 cm^{-1}, which was unchanged by esterification, confirmed that this mixture contained keto and hydroxy free fatty acids. A positive test on fraction II with TBA indicated also the presence of endoperoxides or, at least, compounds which decompose under conditions of the test to give malonaldehyde (3,9).

Fraction II gave 43 bands in the final stage of preparative TLC. Thirty-three bands had definite activity. IR spectra are strikingly similar, exhibiting the same major features as noted for the unseparated fraction II. UV spectra are also similar: absorption at 275 nm and 220 nm, with the latter being strongest. Considering the ease with which polyunsaturated fatty acids can undergo autoxidation, the isolation and separation procedures were modified to minimize exposure to air and light. Band patterns remained unchanged. It therefore appears that these substances are produced by *E. microcarpa*.

Extraction of pond water from which *E. microcarpa* was gathered, followed by the same chromatographic procedures shown in the separation scheme produced many bands in common to those purified from the plant. Commonality was shown also by HPLC. Pond water concentrations of constituents equivalent to fraction II were ca. 0.5 ppm.

The results of the TBA test, IR and chemical evidence suggested that some components of fraction II may be similar to the prostaglandins. Relative retention times of PGA_1 and PGE_1 were compared with components of fraction II by HPLC. Figure 2 shows that elution occurs in the less polar half of the chromatogram.

STRUCTURAL STUDIES

The compound chosen for structural studies, I, (4:6 shown in Fig. 1), was roughly in the middle of those bands having good activity. The HPLC retention time is shown in Figure 2. IR (film) 3400 (broad), 2850 (broad) tailing to 2350, 2925 (s), 2850 (s), 1710 (s) cm^{-1}. IR (methyl ester) (film) 3400 (broad), 2925 (s), 2850 (s), 1735 (s) cm^{-1}. Little difference between I and its methyl ester was noted in the 3400 cm^{-1} region, but the broad absorption between 2850 and 2350 cm^{-1} was eliminated by esterification which confirmed the presence of

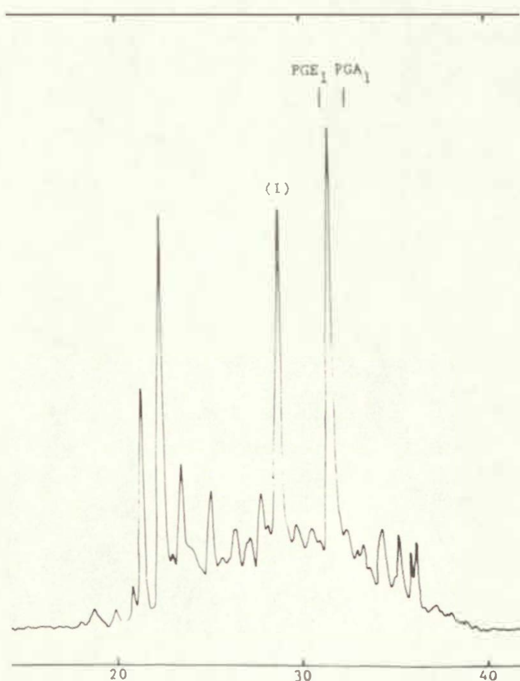

FIG. 2. Analytical HPLC of fraction II. Retention times of PGE_1 and PGA_1 are shown by vertical lines.

a carboxyl group in I. Also, the carbonyl absorption was shifted to 1735 cm^{-1}, which is normal for an isolated ester group. Multiple hydroxyl groups are indicated by the large absorption of the ester 3400 cm^{-1}. We used the method of Albro and Fishbein (10), by which the ratio of the intensity of the 3400 cm^{-1} absorption to that of the ester carbonyl is compared with known esters having different numbers of hydroxyl groups; we calculated the ester of I to have 3 such groups.

Low resolution mass spectrometry (MS) of I produced a complex pattern (Fig. 3) and had several metastable ions that aided in assigning fragmentation pathways. High resolution MS produced empirical formulas for all ions over 69 m/e. Maximum m/e was 316.2045 with a calculated formula of $C_{20}H_{28}O_3$ and 7 rings and double bonds (R+D). Since 3 hydroxyl groups and at least one carboxyl group were indicated from chemical and spectral data and since high molecular weight secondary alcohols can be expected to dehydrate readily on evaporation (11), 2 water molecules were assumed to be lost prior to detection. The trimethylsilyl derivative of I, by GC-MS, showed ion clusters centered at 498 and 572. Since these derivatives give losses of ca. 73 amu (12-14), a 352 MW for I was considered probable.

Ions containing 2 oxygen atoms and one (R+D) corresponding to the end of the molecule having the carboxylic acid group were found as follows: m/e 73, $C_3H_5O_2$; m/e 87, $C_4H_7O_2$; m/e 115, $C_6H_{11}O_2$; m/e 129, $C_7H_{13}O_2$; and m/e 143, $C_8H_{15}O_2$. This series of ions denoted the presence of a group of seven methylenes adjacent to the carboxyl group. The odd electron ion m/e 156, $C_9H_{16}O_2$ was significant when considered in conjunction with m/e 169, $C_{10}H_{17}O_2$, 2 (R+D) in indicating the presence of a double bond between carbons 9 and 10. Ion m/e 199, $C_{11}H_{19}O_3$, 2 (R+D) represents the addition of CH_2O to m/e 169. This addition indicates the presence of a hydroxyl group on carbon 11. Ions at m/e 211, $C_{12}H_{19}O_3$; 3 (R+D) and m/e 223, $C_{13}H_{19}O_3$; 4 (R+D) suggest a triple bond between carbons 12 and 13. The next higher mass ion which contains 3 oxygens is m/e 301, $C_{19}H_{25}O_3$; 7 (R+D) representing the addition of C_6H_6 and 3 (R+D). There are very few possible structures for a C_6H_6 neutral fragment. Since the spectrum shows no ions contaiing 3 oxygen atoms between m/e 223 and 301, the 78 amu loss (C_6H_6) was probably due to a methyl substituted cyclopentadiene. The ion at m/e 301 is also the most abundant high mass ion and is obviously due to loss of a methyl group from the parent ion.

The Δ^9 double bond was confirmed by GC-MS of ozonolysis products from the ester of I which produced 9-oxymethyl nonanoate. Other ozonolysis products were not identified. The above evidence suggests II (see Fig. 4) for the dehydrated 316 MW compound detected by MS.

The 90 MHz ^{1}H-NMR ($CHCl_3$-d) of I, 0.87 ppm (m,3H,$CHC\underline{H}_3$), 1.32.1.53, 1.78 (broad t, 16 H,$nC\underline{H}_2$), 2.14 (s,2H,$CHC\underline{H}_2$), 2.33

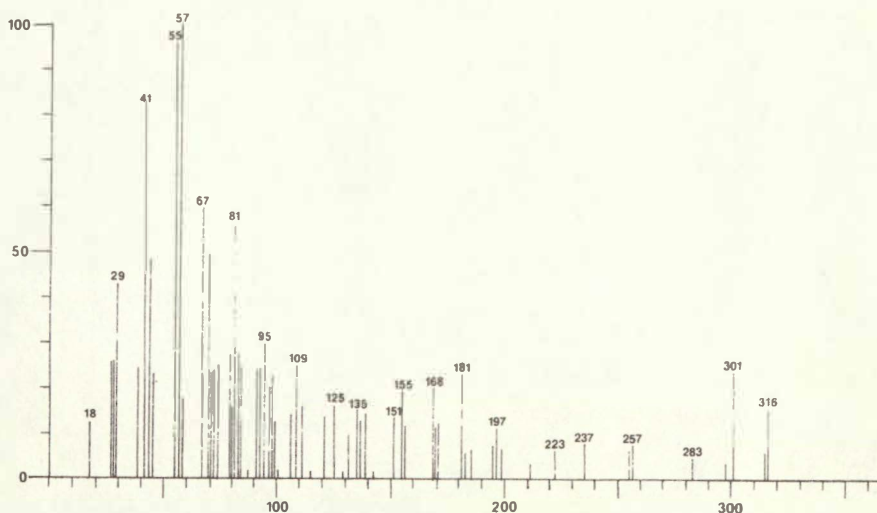

FIG. 3. MS fragmentation pattern of I.

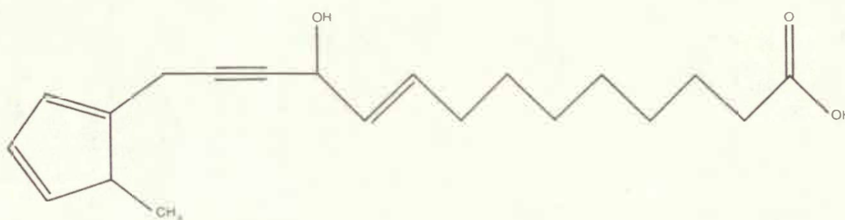

FIG. 4. Structure of II.

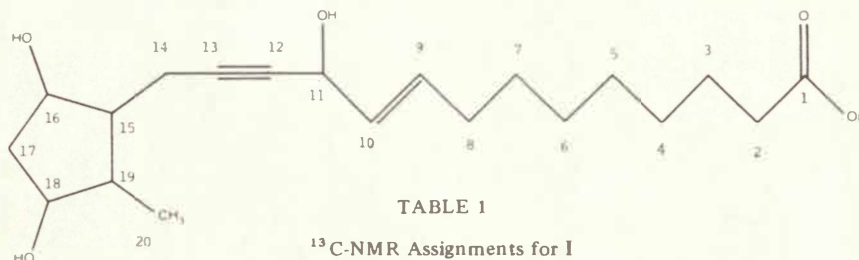

TABLE 1

^{13}C-NMR Assignments for I

Carbon no.	Chemical shifts (ppm)	Carbon no.	Chemical shifts (ppm)
1	162.2	11	56.0
2	34.1	12	82.1
3	24.7	13	83.5
4	29.0	14	29.7
5	29.7	15	52.0
6	29.7	16	77.2
7	24.6	17	41.1
8	34.0	18	78.4
9	116.0	19	43.9
10	132.3	20	14.3

(s,2H,CH_2COOH), 2.90 (broad, 1 H, OH non H-bonding), 3.70 (broad, 2H,CH_2CH OH CH), 4.70 (d,1H,CCH OH CH), 5.42 (broad m, 2H, nCHCH, 7.42 (broad m, 4H,OH), confirms most features indicated by MS. Additionally, the broad absorption at 7.41 ppm shows 4 replaceable protons.

The ^{13}C-NMR spectrum (Table 1) was obtained with 64,000 scans and produced shifts for 20 carbons. We were particularly interested in confirming the existence of the 2 acetylenic carbons indicated by MS and needed information about the ring substitution pattern. Cooper and Fried (15) correlated chemical shifts for several prostaglandin analogs, including acetylenic carbons at approximately the same relative positions. Their acetylenic carbon absorptions averaged 82 and 85 ppm, very close to those obtained for carbons 12 and 13 of I. The 1,2,3,5 substitution pattern of the ring also appears to be confirmed. Cooper and Fried determined shifts for carbons equivalent to C_{16}, C_{17}, and C_{18} in I to be ca. 73, 42 and 78 ppm, respectively. Dehydration of I would be expected to produce the cyclopentadienyl ring indicated in II. We conclude that I has the structure shown in Table 1.

As stated eralier, I is representative of many components in fraction II. In addition, HPLC cochromatography of fraction II with PGA_1 and PGE_1 shows similarity. These components of *E. microcarpa*, therefore, may have physiological properties other than inhibition of blue-green algae.

Further studies are in progress. HPLC is more convenient than TLC and has shown the potential of providing larger amounts for more detailed studies. We have preliminary evidence that many of the same oxygenated fatty acids occur in other aquatic plants. They may, in fact, be widespread in such plants and may partially explain diversity and succession in aquatic systems.

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