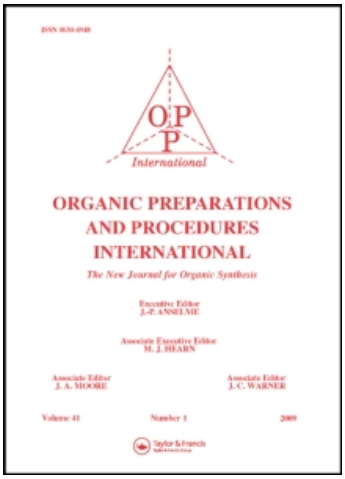

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J. H. Ford[a]; F. A. Mackellar[a]; P. A. Meulman[a]; R. J. Wnuk[a]; G. C. Prescott[a]
[a] The Upjohn Company, Kalamazoo, Michigan

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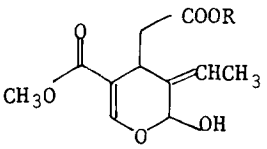
PREPARATION OF CALCIUM ELENOLATE FROM OLIVE PRESS JUICE

J. H. Ford, F. A. MacKellar
P. A. Meulman, R. J. Wnuk, and G. C. Prescott
The Upjohn Company
Kalamazoo, Michigan 49001

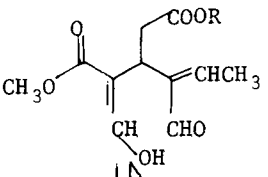
Calcium elenolate is a broad spectrum virucidal agent which is active *in vitro*[1] and *in vivo*[2]. Its preparation from the aqueous press juice of ripe olives is described in a patent[3], but in our hands this method gave products that were only about 10-20% pure. We applied partition chromatography and chromatography on Amberlite XAD-2[4] to the crude elenolic acid and obtained material of 80-90% purity which was further purified by recrystallization of the calcium salt.

A gas liquid chromatographic assay for elenolic acid was developed and used to follow the progress of the purification.

The structure of elenolic acid has been proposed as a mixture of tautomeric forms represented by I, II, and III (R=H),[5,6] whereas the calcium salt has only been represented by I (R=1/2 Ca).[3] On the basis



I



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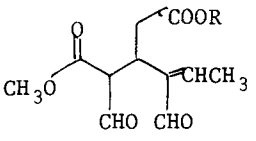

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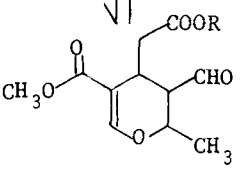
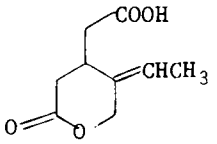


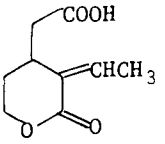
IV

of I.R. and N.M.R. data[7] it is suggested that both elenolic acid and its calcium salt are better represented by IV.[8]

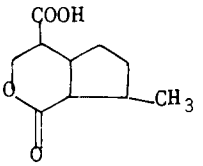
Three crystalline compounds were isolated during purification of elenolic acid. On the basis of spectral data and elemental analyses we propose the following structures:



V



VI



VII

The olive juice came from two different sources in Spain. From the first source we isolated VI and did not observe any V. From the second source we isolated V and did not observe any VI. Compound VII was obtained from a mixture that was derived from both sources.

Experimental

Samples for the GLC method of determining elenolic acid were prepared by dissolving in dioxane, adding palmitic acid as an internal standard, esterifying with an ether solution of diazomethane, evaporating to dryness and redissolving in chloroform. The use of diazomethane as the esterifying agent and dioxane as the solvent was necessary since elenolic acid was degraded under other conditions. Gas chromatography was carried out with a Hewlett-Packard model 402 gas chromatograph fitted with a flame ionization detector. The column was 1.83 m x 3 mm ID glass packed with 3% OV-1 On Gas Chrom Q. An oven temperature of 155°, carrier flow (nitrogen) of 50 ml/min, hydrogen flow of 25 ml/min and air flow of 425 ml/min was

maintained. A sample of 5λ of a 3 mg/ml solution of elenolic acid was injected directly on the column. The range x attenuation was 10 x 16. The method is rapid, causes no observable degradation and separates closely related compounds. The relative standard deviation for the major component is about 3.5%.

Infrared spectra were obtained as Nujol mulls using a Perkin-Elmer model 421 spectrophotometer.

Mass spectra were obtained at 70eV using an Atlas CH-4 mass spectrometer incorporating a TO-4 ion source.

NMR spectra were recorded on a Varian A-60 spectrometer and calibrated with TMS or DDS as internal reference. Spectrum analysis and spin decoupling were done on a Varian HA-100 frequency sweep spectrometer.

Calcium Elenolate

Olive press juice, 13,700 ℓ, was adjusted to pH 2.0 with sulfuric acid and heated to 95° for 3 hrs. After cooling to 60°, 2055 kg of sodium sulfate was added and the solution was cooled to 20° and extracted three times with 4570-ℓ portions of dichloromethane. The combined extracts were concentrated to 3300 ℓ by distillation and extracted with a solution of 27 kg of sodium bicarbonate in 670 ℓ of water. A second extraction was made with 1.3 kg of sodium bicarbonate in 310 ℓ of water. The combined bicarbonate extracts were washed three times with 400-ℓ portions of dichloromethane to remove phenol which had been used to preserve the juice. The washed extract was adjusted to pH 2.0 by cautious addition of sulfuric acid and 136 kg of sodium sulfate was dissolved in the solution. The solution was extracted three times with 310-ℓ portions of dichloromethane. The extracts were combined and concentrated by distillation. The concentrate contained 16 kg of solids

which were 17% elenolic acid by GLC assay.

The solvent system for the partition chromatography had the following composition by volume: 6 toluene, 4 Skellysolve B,[9] 5 acetic acid, 5 water. The packing was prepared by suspending 20 kg. of Dicalite 4200[10] in 160 ℓ of upper phase and adding 8 ℓ of lower phase with rapid stirring. The suspension was added rapidly to the column (40.6 cm x 4.56 m.). This operation was repeated until 100 kg. of Dicalite 420 had been placed in the column. The feed solution contained 4.3 kg of solids, 2.2 kg of acetic acid and 2.1 kg of water. It was added to the suspension of 8.0 kg Dicalite 4200 in 32 ℓ of upper phase. The feed suspension was added rapidly to the column and the column was developed with 2100 ℓ of upper phase. The fractions were pooled on the basis of GLC assays. The main peak contained about 1000 g of solids and 700 g of elenolic acid.

Fifty-three liters of Amberlite XAD-2[4] was stirred with 200 ℓ of boiling methanol, filtered, and washed with methanol. It was then placed in a 15.2-cm diameter column 3.65 m high and washed with 500 ℓ of water. The feed solution contained 1.2 kg of solids from the partition chromatography in 40 ℓ of water. It was passed through the column at 0.33 ℓ/min and followed with a water-40% aqueous acetone gradient, 250 ℓ of each. The eluate was collected in 20-ℓ fractions. The fractions which contained 80-90% elenolic acid by GLC were pooled and concentrated to 16 ℓ under reduced pressure. The resulting solution contained 670 g. of solids.

The 16 ℓ of aqueous elenolic acid solution described above was stirred vigorously at 25° while 450 g of calcium carbonate was added in several portions. A rapid stream of nitrogen was passed over the surface

and the stirring was continued until pH 7.3 was reached (3 hrs.). The calcium carbonate was removed and the filtrate was concentrated to about 5 ℓ at 20-22° in a rotary flask evaporator and stored overnight at 2°. The crystals were centrifuged in a basket centrifuge and the mother liquor was concentrated to obtain a second crop. The moist crystals were combined, dissolved in water at 25°, decolorized with Darco G-60[11] and crystallized as before. The crystals and filtrate were freeze dried. The yield of first crop crystals was 253 g; m.p. 164° (dec.); $[\alpha]_D$ -169°[12] (water, 10 g/ℓ); U.V.: λmax. (water), 239 nm (ε 22,100).[12]

Anal. Calc'd for $C_{22}H_{26}O_{12}Ca$: C, 50.57; H, 5.02; Ca, 7.67; eq. wt., 261. Found:[12] C, 50.61; H, 5.49; Ca, 7.91; eq. wt., 261; IR (Nujol): 3460 (H_2O), 1705 (>C=O, ester & aldehyde), 1625 (C=C) 1555 & 1420 (carboxylate ion), 1290, 1195, 1110, 1060 cm^{-1}.

NMR (D_2O); δ = 1.60 (d, 3,J=7.0 Hz, C-methyl), 2.22 (A of ABC, 1, J_{AB} = 15.0 Hz, J_{AC} = 11.0 Hz, α-acid hydrogen), 2.72 (B of ABC, 1, J_{BC} = 3.5 Hz, α-acid hydrogen), 3.32 (C of ABC, 1, J_{CD} = 0.5 Hz, β-acid hydrogen), 3.82 (s., 3, O-methyl), 4.47 (bq, 1, J = 7.0 Hz, α-ether hydrogen), 7.73 (bs, 1, vinyl hydrogen), 9.77 (bs, 1, aldehyde hydrogen). NMR (H_2O) same as above except aldehyde hydrogen was a doublet J = 2.0 coupled to the new α-aldehyde hydrogen, broad unrelated singlet at 2.9δ.

Compound V

Compound V was prepared by counter current distribution of an elenolic acid concentrate (before partition chromatography), using the system dichloromethane-water. After 400 transfers in a 200-tube, 50-ml-per-phase Craig machine the contents of tubes 110-199 were concentrated to an oil which solidified. It was recrystallized from ethyl acetate and then from hot water using Darco G-60 to give colorless crystals;

m.p. 91-94°; $[\alpha]_D$ -0.1° (water, 10 g/ℓ).

Anal. Calc'd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57; eq. wt., 184. Found: C, 58.78; H, 6.62; eq. wt., 182; IR (Nujol): 3000 (OH), 1732 (>C=O, lactone), 1695 (>C=O, acid), 1670 (C=C), 1324, 1275, 1245, 1186, 1173, 1155, 1090, 1008 $cm.^{-1}$.

Mass Spectrum: 184 (M^+), 169, 166, 155, 138, 125, 124.

NMR ($CDCl_3$): δ 1.73 (d, 3,J= 7.0, vinyl methyl), 2.49 (A of ABMXY, 1, J_{AB} = 16.0, J_{AM} = 10.0, α-acid hydrogen), 2.62 (B of ABMXY), 1, J_{BM} = 2.0, α-acid hydrogen), 2.70 (X of ABMXY, 1, J_{XY} = 16.0, J_{MX} = 8.0, cyclic α-keto hydrogen), 2.80 (Y of ABMXY, 1, J_{MY} = 4.0, cyclic α-keto hydrogen), 3.34 (M of ABMXY, 1, tertiary allylic hydrogen), 4.56 (A of AB, 1, J_{AB} = 13.0, allylic lactone hydrogen), 4.81 (B of AB, 1, allylic lactone hydrogen), 5.68 (ba. J = 7.0, 1, vinyl hydrogen), 11.2 (s. 1, carboxyl hydrogen).

Compound VI

Compound VI crystallized from an elenolic acid concentrate (before partition chromatography) during storage at 5°. The crude crystals were washed with acetone and recrystallized twice from hot water; m.p. 153-154°; $[\alpha]_D$ -129° (water, 10 g/ℓ); UV: λmax. (water) 223 nm (ε 7030).

Anal. Calc'd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57; eq. wt., 184. Found: C, 58.74; H, 6.82; eq. wt., 183; IR (Nujol): 3090 (OH), 1725 (>C=O, lactone), 1678 (>C=O, acid), 1622 (C=C), 1351, 1276, 1196, 1185, 1167, 1035 cm^{-1}.

NMR (d_6 DMSO): 1.86 (d, 3, J = 7.0, vinyl methyl), 2.43 (d, 2, J = 7.2, α-acid methylene), 3.37 (broad quintet, 1, J = 7.0, tertiary allylic hydrogen), 4.15 (A of ABXY, 1, J_{AB} = 11.5, J_{AX} = 6.0, J_{AY} = 4.0, α-ether hydrogen), 4.37 (B of ABXY, 1, J_{BX} = 8.5, J_{BY} = 4.0, α-ether hydrogen),

6.87 (dq, 1, J_{AX_3} = 7.0, $^4J_{AM}$ = 1.5, vinyl hydrogen).

Compound VII

Compound VII was isolated from dextrorotatory mother liquor solids that had been obtained by the crystallization of calcium elenolate after partition chromatography. After 195 transfers in a 200-tube Craig machine using the system 5 dichloromethane, 4 water, 1 acetic acid, the contents of tubes 90-115 were evaporated to dryness. The resulting white crystalline residue was recrystallized from acetone and then from hot water; m.p. 185-188°; $[\alpha]_D$ +105° (ethanol, 10 g/ℓ); UV: max (ethanol) 214 nm (ε 1550).

Anal. Calc'd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12; eq. wt., 198. Found: C, 60.11; H, 7.20; eq. wt. 197.

Mass Spectrum: 198 (M^+), 183 (M^+-CH_3), 180 (M^+-H_2O), 156 (M^+-CH_2CO), 152 (M^+-H-COOH), 143, 109.

IR (Nujol): 3000 (OH), 1738 (>C=O, lactone), 1684 (>C=O, acid), 1349, 1321, 1257, 1211, 1202, 1077, 1027 cm^{-1}.

NMR (d_6 DMSO): 1.95 (d, 3, J = 5.5, methyl), 2.40 (t, 1, J = 9.0, α-keto hydrogen), 2.85 (m, 1, tertiary β-acid hydrogen), 3.00 (m, 1, tertiary α-acid hydrogen), 4.25 (d, 2, J = 8, α-lactone methylene).

Compound VII was also isolated by partition chromatography from the last fractions in the elenolic acid peak.

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7. R. C. Kelly and F. A. MacKellar, to be published.

8. It has been suggested that a small equilibrium concentration of I, II, and III may be admixed with IV. Private Communication, Dr. R. C. Kelly.

9. A purified hexane fraction supplied by Skelly Oil Co.

10. A diatomaceous earth supplied by Great Lakes Carbon Co.

11. An activated carbon supplied by Atlas Chemical Industries.

12. Corrected for 1.61% water.

13. Other lots with essentially the same elemental analyses and spectra gave specific rotations from -162° to -175°.

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