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# Bioactive compounds from the alga *Dictyopteris undulata*

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**Abstract**
An investigation of biologically active compounds from the alga *Dictyopteris undulata* by bioassay-guided fractionation has led to the isolation and identification of zonarol, chromazonarol, zonaroic acid and the hitherto unreported isozonaroic acid. Their structures were determined by chemical transformation, GC and GC- MS comparison of transformed intermediates and by spectroscopic means. X-ray crystallography of zonarol confirmed the gross structure of this compound and also gave the relative stereochemistry at C- 9 and C- 10 as *trans.* All of these compounds were found to exhibit antimicrobial activity. Some also showed activity against L1210 cells and antiviral activity.

*Keywords*: Bioactivity-guided isolation; Antimicrobial; Antiviral; L1210 cells

## Introduction

Brown marine algae of the Dictyotaceae family among marine algae are a rich source of secondary metabolites with novel structures and desirable activities (Rinehart *et al.,* 1981). Fairly extensive studies have been made on the chemical composition of *Dictyopteris undulata.* One of the first compounds isolated from this species was the hydrocarbon zonarene 1, followed by zonarol 2 and isozonarol 3 (Fenical *et al.*, 1973). Zonarol 1 was the exclusive isomer obtained from *D. undulata* sample collected in the Pacific Ocean while isozonarol 3 was the only isomer obtained from *D. undulata* sample collected in the Gulf of California. The total synthesis of these latter two compounds has been achieved (Welch *et al.*, 1978). Isolated as minor constituents from this species were chromazonarol 4 and isochromazonarol 5 (Fenical *et al.*, 1975). It is worth noting that the enantiomeric chromazonarol 6 has been reported to have been isolated from the sponge *Disidea pallescens* (Cimino *et al.*, 1975). Another sesquiterpenoid component zonaroic acid 7 has been obtained from *D. undulata* (Cimino *et al.*, 1975). From the methanolic extracts of fresh *D. undulata* collected in the Bay of Tosa, Japan, has been isolated 2, 3 and 7 as well as a compound named yahazunol 8 (Ochi *et al.*, 1979). The structures of zonarol 2 and isozonarol 3 were determined (Fenical *et al.*, 1973) from spectroscopic data, derivatisation, chemical transformation and degradative studies of these starting materials. The same

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methods were employed to characterise chromazonarol 4 and isochromazonarol 5 (Fenical *et al.*, 1975). The structure of zonaroic acid 7 was determined from spectrospic data, derivatisation, degradative and gas chromatographic studies (Cimino *et al.*, 1975). Comparison of the optical rotation of the degradative products obtained from the degradation of ambrein and manool led to deducing the stereochemistry of zonarol and zonaroic acid as 5R, 9R, 10S. The sesquiterpene quinols 9, 10 with a skeletal structure similar to compounds isolated from *D. undulata* have been isolated from the Micronesian sponge *Aka* species (Mukhu *et al.*, 2003). Zonarol and isozonarol were found to be fungitoxic towards certain pathogens (Fenical *et al.*, 1973). These compounds along with zonaroic acid, yahazunol, were found to exhibit moderate antimicrobial activity (Ochi *et al.*, 1979).

In a search for new bioactive compounds, a study was undertaken to isolate and characterise bioactive compounds from *Dictyopteris undulata* (johnstonii) by bioactivity-guided fractionation.

## Experimental

*General.* Melting points were determined on a Thomas-Hoover apparatus or Kofler hot stage, are uncorrected, and are given in degrees centigrade. Optical rotations were measured on an Autopol III automatic polarimeter Infrared (IR) spectra were recorded on a Beckman IR 12 spectrophotometer, either in solution, in nujol, in a potassium bromide pellet or neat. Ultraviolet (UV) spectra were obtained using a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer.

Proton magnetic resonance ($^1$H NMR) spectra were determined on Varian EM 390, Varian HR-220 and Nicolet NTC360 spectrometers using tetramethysilane (TMS) as an internal standard. Carbon magnetic resonance ($^{13}$C NMR) spectra were recorded on JEOL FX-60, Varian XLFT-100 and Nicolet NTC 360 instruments at 15MHz, 25.2 MHz and 90.6 MHz respectively, using various deuterated solvents.

Low resolution electron impact (EI) spectra were determined on a Finnigan MAT CH-5 spectrometer. Low resolution gas chromatography/mass spectrometry (GC/MS) was performed on a Varian 1700 gas chromatograph coupled to a Finnigan MAT 311A spectrometer. Field desorption (FD) and high resolution electron impact (HREI) mass spectra were determined on a Finnigan MAT 731 spectrometers. Chemical ionization (CI) and fast atom bombardment (FAB) mass spectra were obtained on 311A or VG Analytical 7070 mass spectrometers. X-ray crystallographic data were obtained by the X-ray Laboratory, School of Chemical Sciences, University of Illinois.

Gas chromatography (GC) was performed on Varian 1700 and 3700 chromatographs. High pressure liquid chromatography (HPLC) was performed on Waters Associates instruments equipped with a variable wavelength detector and an Altex Ultra Sphere ODS column (25 cm length, 8 cm internal diameter 5 µ mesh).

Thin layer chromatography, TLC, was carried out on Brinkmann polygram SIL G/$UV_{254}$ (0.25 mm or 2 mm) and on Whatmann $KC_{18}F$ reversed phase 200) TLC plate. Compounds were detected under a UV lamp or by the use of the appropriate spray reagent.

Column chromatography was carried out with silica gel (Brinkmann, 50-200µ), Sephadex LH-20 (Pharmacia, 25-100µ) or CHP-20P Resin (Mitsubishi, 55-150µ) with a stationary phase compound ratio at least 100:1. Glass wool was placed in the bottom of the column just above the stopcock and the column was partially filled with the appropriate solvent. Slurry of the stationary phase was allowed to settle while the column

was tapped gently to ensure even parking as the solvent flowed out.

*Bioautography.* A TLC plate of a crude sample or relatively pure fraction was run in an appropriate solvent system. After allowing solvent to evaporate at room temperature, UV-active spots were marked under the UV lamp and the plate was placed face down on microorganism seeded agar in a Petri dish. The spots were also marked on the Petri dish and the Petri dish was placed in a refrigerator (4° C) for an hour (so that all components on the TLC plate were absorbed onto the agar, after which the TLC plate was removed from the Petri dish and the latter was transferred to an incubator (35° C) for 12-24 hr. A zone of inhibition was observed around any spot that had an antimicrobial component.

*Alga collection.* The alga *D. undulata* (johnstonii) was collected at a depth of 1.5 to 8.5m off shore from the Santa Catalia Marine Science Center, California (118.29°, W longitude, 33.26°, N latitude) and stored in plastic bags at -20° C.

*Extraction. D. undulata* (4.14kg, wet weight) was cut into small pieces and allowed to air dry. The air dried sample was extracted several times with methanol-toluene (3:1) until the filtrate was colourless. The solvent was removed under reduced pressure to give a greenish-brown material. This was dissolved in chloroform and filtered to desalt the extract. The solvent was then removed under reduced pressure to obtain a greenish-brown material.

*Column chromatography of D. undulata.* Following bioautography the crude desalted *D. undulata* extract (48g) was dissolved in a minimum amount of ether-petroleum ether (20:80) and applied to a silica gel column (1.75kg; 15 x 125cm) prepared in the same solvent system. The column was eluted successfully with 20% (4.5L), 60% (9L), 70% (3.6L), 80% (5.5L) and 90% (5.5L) ether in petroleum ether; then with pure ether (7.25L) and 10% (5.5L) methanol in ether. Thirty-one fractions were collected, not necessarily of the same volume. Following biological activity tests of all the fractions against *B. subtilis, E. coli, S. cerevisiae* and *P. atrovenatum,* they were appropriately combined into five larger fractions, Fractions 1-5. Other biological activity tests were performed on these combined fractions.

*Isolation of zonarol.* Combined fraction 3 (19.70g) was dissolved in a minimum amount of benzene and applied to a silica gel column (1.0kg; 15 x 125cm) prepared in a 10% ether in benzene. The column was then eluted successively with 10% (4.6L), 20% (4.6L), 30% (2.3L), 60% (2.3L) and 75% (2.30L) ether in benzene. The fractions collected were not necessarily of equal volume. A solid material was obtained in most of the fractions after removal of solvent. These solids were combined and purified by repeated recrystallisation from ether-petroleum ether. Final recrystallisation was performed in carbon tetrachloride to give zonarol (2.31g, 0.04% form wet alga).

*Isolation of chromazonarol.* After bioautography of a sample of combined fraction 2 in 30% ether in benzene, 40% ether in benzene, 50% chloroform in hexane, 50% chloroform in petroleum ether, 40% ethyl acetate in hexane and 50% ethyl acetate in hexane, 2.86g of this combined fraction was dissolved in a minimum amount of 20% ethyl acetate in hexane and applied to a silica gel column (500g, 13.5 x 85cm) prepared in the same solvent system. The column was eluted successively with 20% (1.75L), 25% (4.60L), and 30% (2.30L) ethyl acetate in hexane. The fractions collected were not necessarily of the same volume. After biological testing against *B. subtilis*, the latter fractions obtained from the runs were combined and purified further by preparative TLC in 90% chloroform in hexane to give a glassy product (0.457g, 0.008% based on wet weight alga) on drying under vacuum. The pure compound was

tested for biological activity. This compound was identified as chromazonarol.

*Isolation of zonaroic and isozonaroic acids.* Antibacterial activity having been ascertained in Fraction 4, the fraction was bioautographed in the following solvent systems: 30% and 40% ether in benzene, 25% ether in carbon tetrachloride, 90% chloroform in hexane, 90% chloroform in petroleum ether, 20% and 25% ether in benzene. Fraction 4 was dissolved in a minimum amount of ether and applied to a silica column (600g, 13.5 x 85cm) that had been prepared in 30% ether in benzene and the column was eluted successively with 30% (3.60L), 35% (3.60L), 40% (3.60L) and 45% (1.80L) ether in benzene. Following removal of solvent from the fractions collected, each was tested for bioactivity. The latter fractions which showed bioactivity were combined. A small portion of this was subjected to preparative TLC in 35% ether in benzene which was worked up to give a solid product. Although this gave a single spot on TLC, spectroscopic data ($^1$H NMR) indicated it was a mixture of compounds. Reversed phase HPLC of the combined bioactive fraction was effected in order to separate the mixture into pure compounds. Methanol-water (85.15) HPLC in 0.01 N sodium acetate in acetic acid, pH4.60, (flow rate 1.5-mL/min, chart speed 40cm/hr, UV detector at 250nm) on the Waters instrument gave separation. On the Altex instrument the conditions for separation were: 85% MeOH/$H_2O$, NaOAc buffer at pH 4.6, flow rate 14-mL/min, chart speed 30 cm/hr, 254 UV detector.

Solvent was removed from the sample peaks to give a whitish product. The sodium acetate was removed by dissolving this product in chloroform, filtering and retaining the supernatant. Chloroform was removed under reduced pressure to give a colourless semi-crystalline product.

The more polar of the compounds isolated from the mixture (0.3085g, 0.006% based on wet weight of alga) was identified as zonaroic acid.

The less polar of the two compounds, separated by HPLC of the mixture obtained from silica gel column chromatography of Fraction 4 (0.377g, 0.0073% based on wet weight alga) was identified as isozonaroic acid (11).

## Results

This alga was collected by SCUBA techniques near Catalina, California, U.S.A. The crude extracts of this alga indicated it was cytotoxic (16mm zone at 200µg/disc, 15 mm at 100µg, 13 mm at 50µg) but showed no *Herpes simplex* virus type 1 (H S V-1) inhibition. Antimicrobial activity runs indicated this alga possessed a trace of activity against *B. fragilis* and *C. perfringens*. *D. undulata* also showed a trace of activity-at full strength against *C. fuscicalata* and *T. pyriformis* in the antiprotozoal assay. Following extraction at room temperature with methanol/toluene (3:1) and bioautography, the crude extract was subjected to silica gel chromatography using gradients of ether in petroleum ether followed by gradients of methanol in ether to give smaller fractions each of which was assayed for antimicrobial, antifungal activity then combined to give five larger fractions. The third combined fraction (Combined Fraction 3) which contained a solid material was subjected to extensive silica gel column chromatography in ether-benzene to yield a colourless crystalline compound.

The EI mass spectrum of this compound gave a molecular ion peak at $^m/_z$ 314 ($C_{21}H_{30}O_2$), The $^1$H NMR spectrum showed three protons as a multiplet at δ 6.60 (aromatic), two hydroxyl protons at δ 4.5, $^{13}$C NMR signals at δ 148.3 and 150.5. Two terminal methylene olefinic protons were observed at δ 4.70, 4.80 and a doublet at δ 2.70 (benzylic protons) as well as $^{13}$C NMR shifts at δ 108, 129.86 and 26. A series of

multiplets were observed between δ 2.5 and 1.10 as well as several overlapping singlets at δ 0.85 (Table 1).

Other significant peaks in the mass spectrum were observed at $^{m}/_{z}$ 191 (M – $C_7H_7O_2$) and $^{m}/_{z}$ 129 ($C_7H_7O_2$).

The IR and UV of this compound were also obtained (See Experimental Section). Crystals of this compound were subjected to X-ray crystallographic analysis as crystals of suitable derivatives could not be obtained and is presented (Fig. 1).

The results obtained indicated the gross structure of this compound and gave the relative stereochemistry at C-9 and C-10 as *trans*.

Combined fraction 2 on being subjected to silica gel chromatography followed by purification by preparative thin layer chromatography yielded a semi-solid biologically active compound. The high resolution FAB mass spectrum of this compound gave a molecular ion at $^{m}/_{z}$ 315.2257 ($C_{21}H_{31}O_2$) and the EIMS a molecular ion at $^{m}/_{z}$ 314 which is the same value as, hence isomeric with the biologically active compound obtained from combined fraction 3. The $^{1}$H NMR displayed peaks at δ 6.64, 6.61, 2.58 and $^{13}$C NMR signals at δ 11, 116.39, 114.80 and 23.02 indicated similarity in structure. There were however differences in some of the NMR data with $^{1}$H NMR signals at δ1.17, 21.22, and 77.36 for $^{13}$C NMR (Table II). Signals at δ 0.90, 0.88 and 0.84 and multiplets between δ 2.0 and 1.20 were also observed in the $^{1}$H NMR. The EI mass spectrum of this compound showed prominent peaks at $^{m}/_{z}$ 191 and 123.$^{13}$C NMR indicated a peak at δ 77.36. A comparison of the chemical shifts and off resonance multiplicities for compound obtained from this fraction to that obtained from Combined Fraction 3 indicated differences in structure at one carbon atom. Compound obtained from Fraction 3 on treatment with p-toluene sulphonic acid in benzene gave a product whose $^{1}$H NMR $^{13}$C NMR and $R_f$ values were identical to the natural product obtained from Combined Fraction 2 (See Experimental Section). This synthetic compound was converted to the acetate ($Ac_2O$ in pyridine) which was found to be very similar to the acetate of compound obtained from Fraction 2 prepared under the same conditions, with respect to their $^{1}$H NMR, FAB mass spectra and $R_f$ values.

Combined Fraction 4 from this alga on being subjected to repeated silica gel chromatography followed by preparative TLC gave a gummy product which showed a single spot on TLC but was found to be a mixture of compounds from $^{1}$H NMR data. Reversed phase HPLC using MeOH/$H_2O$ (17:3) in a NaOAc-HOAc buffer (0.1M pH 4.6) of this mixture yielded two semi-solid products. The more polar compound had $^{1}$H NMR signals at δ 7.83, 7.78 and 6.78 (aromatic protons), at δ 4.82, 4.68 (olefinic acid 2.70 (benzylic) and corresponding $^{13}$C NMR peaks at δ 129.57, 132.40, 115.10, 121.48, 107.55 and 23.46 respectively. Other $^{1}$H NMR peaks at δ0.94, 0.84, 0.83 (methyl) and between δ 2.4 and 1.0 (saturated methylene and methine groups) were observed.

The IR had peaks at 1680 $cm^{-1}$ (conjugated carbonyl) at 1600 $cm^{-1}$ (aromatic ring) corroborated by a $^{13}$C signal at δ 171.79 and a peak at 900 cm-1 (exocyclic double bond). The mass spectrum showed a molecular ion at $^{m}/_{z}$ 342 ($C_{22}H_{30}O_3$) with the base peak at $^{m}/_{z}$ 191 and another prominent peak at $^{m}/_{z}$ 151. (Table III)

The second and less polar compound isolated from this mixture had mp 107°, $[\alpha]^{25}_{D}$ 17.05 (c 1.14 $CHCl_3$) and UV maxima at 252 nm (ε 8311). Its high resolution EIMS (342.2189, $C_{22}H_{30}O_3$) was found to be isomeric with the more polar compound. The IR showed bands at 3600-3300 $cm^{-1}$ and 1690 $cm^{-1}$ and $^{1}$H NMR signals at δ 7.80 and 6.79 as well as a peak at $^{m}/_{z}$ 151 ($C_8H_7O_3$, HREIMS) in EI mass spectrum all pointing to

a hydroxybenzoic acid group. Other major peaks in the mass spectrum were at $^{m}/_{z}$ 191 (M-151) and 109 (base peak). IR peaks at 2920 and 1280 cm-1 (saturated unit), a peak at 780 $cm^{-1}$ and a one proton broad singlet at δ 5.4 (trisubstituted double bond) were observed. Other proton NMR signals were a two proton doublet at δ 2.65 (benzylic), two proton doublet of doublets at δ 2.04, one proton singlet at δ 2.50 and a three proton singlet at δ 1.43 (vinyl methyl). A number of saturated methylene protons occurred between δ 1.4 and 0.95 and methyl singlets between δ 0.95 and 0.80 indicated methyl groups attached to quaternary carbon atoms. Irradiation of the signals at δ 2.04 and 2.50 caused the singlets at δ 5.4 to sharpen considerably. The $^{13}C$ NMR (Table IV) showed peaks at δ 171.65, 129.41, 122.68, (hydroxybenzoic acid moiety), δ 23.79 (benzylic), 25.99 (allylic), 115.18 (vinylic), 22.38 (vinylic methyl), 121.60 (tetrasubstituted carbon) (Table IV).

This less polar compound was reduced ($H_2/PtO_2$) to yield a product whose FAB mass spectrum showed a peak at $^{m}/_{z}$ 345 (M + H). Reaction of this product with diazomethane followed by capillary GC gave two major peaks. Two minor also were observed. The GC-MS of the major peaks each gave major ions at $^{m}/_{z}$ 358 ($M^+$), 327 (M-$OCH_3$), 193 ($C_9H_9O_3$) and 165 ($C_9H_9O_3$). The two minor peaks each gave major ions at $^{m}/_{z}$ at 372 ($M^+$) and 341 (M-$OCH_3$). HPLC of a hydrogenation product of this compound (MeOH/$H_2O$) 17:3 in NaOAc-HOAc pH4.6) yielded two peaks with retention times 26.7 and 29.4 min.

The more polar compound was subjected to hydrogenation under conditions similar to that done for the less polar compound followed by reaction of the product obtained with diazomethane. Capillary GC gave two major peaks and two minor peaks. The GC-MS of the major peaks each gave major ions at $^{m}/_{z}$ 358 ($M^+$), 327 (M-$OCH_3$), 193 ($M^+C_9H_9O_3$) and 165 (M-$C_9H_9O_3$). HPLC of the hydrogenation product under conditions identical to those employed for the hydrogenation product of this compound yielded two peaks with retention times 26.7 and 29.4 min. Co-injection of the hydrogenation product from each compound and HPLC gave two peaks with retention times 21.6 and 22.6 min.

The biological activities of the isolated compounds are listed in Table V.

**Properties of zonarol**

mp 177-179°; $[\alpha]^{25}_{D}$ + 14.92° (c 1.14 $CHCl_3$);

UV (MeOH), $\lambda_{max}$293nm (ε3400); IR ($CHCl_3$) 3620, 2960, 1650, 1510, 1180, 900$cm^{-1}$;

$^1H$ NMR ($CDCl_3$) δ 6.59 (m, 2H), 6.52 (dd, 1H J = 8.53, 2.0), (6.5 1H, J = 8.53, 2.0), 4.80 (s, 1H), 4.70 (s 1H), 4.52 (s, 1H) and 4.38 (s, 1H), both exchangeable with $D_2O$, 2.71 (d, 2H, J = 6.3), 2.4 – 1.0 (m, 12H), 0.89 (s, 3H), 0.81 (s, 3H);

$^{13}C$ NMR ($CD_3$ $COCD_3$) δ 150.60 (s), 129.86 (s), 116.76 (d), 115.76 (d), 112.85 (d), 108.85 (t), 56.66 (d), 56.26 (d), 42.81 (t), 40.40 (s), 39.68 (t), 38.74 (t), 34.08 (s), 33.95 (q), 25.08 (t), 23.90 (t), 22.06 (q), 20.04 (t), 14.95 (q);

EIMS 315 (8% of base peak), 314 (32, $M^+$), 299 (3), 192 (16), 191 (100), 178 (18), 163 (15), 161)36), 137 (15), 135 (20), 123 (66), 121 (29), 109 (38), 107 (20), 95 (50), 81 (32), 69 (37), 67 (18) 55 (28), 43 (13), 41 (37).

The above data are consistent with those reported for zonarol.

**Properties of chromazonarol**

mp 129-130°; $[\alpha]$ $^{25}_{D}$ – 48.7° (c 1.0 in $CHCl_3$); UV (MeOH) $\lambda_{max}$297 nm (2780), 299 (ε 2740), 220 nm (3017);

IR (neat) 3420 – 3420, 2950, 1620, 1500, 1250, and 940 $cm^{-1}$;

$^1H$ NMR ( $CDCl_3$) δ 6.60 (s, 1H), 6.57 (d, 1H J = 7.2) and 6.54 (dd, 1H J = 6.6, 2.75) all due to aromatic protons, 0.9 (s, 3H), 0.87 (s, 3H) 9.48 (s, 3H);

$^{13}C$ NMR ($CDCl_3$) δ (multiplicities) 149.17 (s), 147.47 (s), 123.82 (s), 118.09 (d), 116.39 (d), 114.80 (d), 77.36 (s), 56.20 (d), 52.58 (d), 42.34 (t), 41.61 (t), 39.69 (t), 37.31 (s), 33.97 (q), 33.72 (s), 23.02 (t), 22.15 (q), 21.22 (q), 20.28 (t), 19.05 (t), 15.37 (q),

EIMS $^{m}/_{z}$ 315 (13), 314 (57), 299 (4), 192 (16), 191 (100), 178 (17), 161 (27), 123 (34), 109 (20), 74 (34), 69 (24), 59 (54), 55 (22), 45 (47), 43 (19), 41 (45), HRFABMS $^{m}/_{z}$ 314.2257 ($C_{21}H_{30}O_2$); Calcd 314.2246

[α] $^{25}_{D}$ – 50 (c 1.0, $CDCl_3$); UV (MeOH), 298 (3900), 228 (6100), 219 (7300); IR 3345; $^{1}$H NMR ($CDCl_3$) δ 6. 55 (3H), 4.55, 2.55. 2.05, 1.16, 0.91, 0.89, 0.86; $^{13}$C NMR ($CDCl_3$) δ148.7, 146.6, 146.6, 123.2, 117.3, 116.0, 114.4, 76.9, 56.0, 52.0, 41.8, 42.0, 39.1, 36.7, 33.3, 33.1, 22.4, 21.5, 20.6, 19.7, 18.5, 14.7; EIMS $M^+$ 314].

**Chromazonarol acetate from natural chromazonarol**

A solution of chromazonarol (6mg; 0.02 millimol) in 0.5ml of pyridine was stirred while 0.5ml of acetic anhydride was added, then stirred at room temperature for three hours. Solvent was removed to yield the product (6.2mg; 90%); $R_f$0.56.

$^{1}$HNMR ($CDCl_3$) δ 6.74 (m, 3 H), 2.61 (d, 2H, J = 9.9), 2.26 (s, 3H) 1.19 (s, 3H), 0.90 (s, 3H) (s, 3H), 0.87 (s, 3H), 0.84 (s, 3H);

FABMS $^{m}/_{z}$ 357 (95) 356 (100), 314 (70), 279 (34), 191 (51), 165 (33), 149 (28), 135 (32), 119 (31), 109 (14),

HRFABMS $^{m}/_{z}$ 315.2220 ($C_{21}H_{31}O_2$); Calcd 315.2246.

**Chromazonarol acetate from semisynthetic chromazonarol**

Semisynthetic chromazonarol (7.9mg; 0.025 millimol) dissolved in 0.5ml of pyridine was stirred, acetic anhydride (0.5ml) was added and the mixture was stirred for 3 hr, after which it was evaporated to give the product (8.2mg; 92%), which had the following properties: $R_f$ 0.56;

$^{1}$H NMR ($CDCl_3$) δ 6.76 (m, 3H), 0.88 (s, 3H), 0.85 (s, 3H);

FABMS $^{m}/_{z}$ 357 (100), 356 (98), 314 (64), 279 (33), 191 (48), 165 (38), 149 (31), 135 (36), 119 (37), 109 (12);

HRFABMS $^{m}/_{z}$ 357.2323 ($C_{23}H_{33}O_3$); Calcd 357.2351.

**Properties of zonaroic acid**

mp 104°; [α] $^{25}_{D}$ +26.83° (c 1.016 in $CHCl_3$); UV (MeOH) $\lambda_{max}$252.3nm (8000);

IR (KBr) 3550-3300, 2940, 1600, 1275, and 900 $cm^{-1}$;

$^{1}$H NMR ($CDCl_3$) δ 7.82 (s, 1H), 7.79 (d, 1H J = 8), 4.81 (s, 1H), 4.68 (s, 1H), 2.78 (d, 2H, J = 5), 2.0-1.0 (saturated methylenes), 0.89 (s, 3H), 0.83 (s, 3H), 0.82 (s, 3H);

$^{13}$C NMR ($CDCl_3$) δ (multiplicities) 171.79 (s), 158.66 (s), 148.75 (s), 132.40 (t), 40.21 (s), 39.15 (t), 38.12 (t), 33.62 (s), 33.62 (q), 24.40 (t), 21.74 (q), 19.42 (t), 14.52 (q);

ENIMS 343 (5), $^{m}/_{z}$ 342 $M^+$, (18), 327 (29), 245 (7), 231 (9), 203 (11), 192 (14), 191 (100), 178 (26), 177 (21), 151 (22), 137 (73), 123 (24), 109 (28), 107 (16), 95 (46), 81 (30), 74 (15), 69 (19), 59 (22), 45 (38), 43 (48), 31 (69).

**11-Zonaroic acid methyl ester**

To 40µg of zonaroic acid in chloroform a few drops of diazomethane were added at room temperature and the solution was allowed to stand for 15min. Capillary GC of the product was performed on an SP 2100 column (10m long, split injection ration of 10:1, helium flow rate 2mL/min, head pressure 10 psi, chart speed 76 cm/hr). A peak with retention time 12.75 min gave the following EIMS data: $^{m}/_{z}$ 356 (30), 341 (31), 314 (31), 271 (8), 259 (9), 245 (11), 232 (7), 220 (15), 217 (18), 191 (100), 177 (19), 165 (78), (60), 121 (30), 109 (45), 95 (49), 81 (44), 55 (35), 41 (42).

**Hydrogenation of zonaroic acid**

Zonaroic acid (2mg) was dissolved in methanol (5mL), platinum oxide (5mg) was added and the flask was placed in a hydrogenation apparatus. Hydrogen was passed through the stirred reaction mixture for 4 hr, after which the solution was filtered and the solvent was evaporated under reduced pressure to give the product in quantitative yield; FABMS $^{m}/_{z}$ 345 (M+H) HRFABMS 345.2452 $C_{22}H_{33}O_3$ (M+H).

HPLC of the reduction product of zonaroic acid was performed on a reversed phase column using 85% MeOH/$H_2O$ in 0.01 N buffer (NaOAc/HOAC pH 4.6 flow rate 3mL/min. chart speed 30 cm/hr, detector 254 nm). Two peaks with retention times 26.7 min and 29.4 min. were observed.

**Dihydrozonaroic acid methyl ester**

To 80µg of the reduction product from zonaroic acid in chloroform were added a few drops of diazomethane at room temperature and the product was allowed to stand for 10 min. Capillary GC and GC/MS of the ester of the reduction product was performed on an SP 2100 column, (10m long, split ratio 10:1, helium flow rate 2mL/min, head pressure 10 psi, chart speed 76 cm/hr). Two major peaks with retention times 15.92 min. 16.21 min. and minor peaks (5% of the former) with retention 16.86 and 17.88 min were observed. The major component with retention time 15.92 min gave the following EIMS peaks: $^{m}/_{z}$ 358 (11, $M^+$), 343 (4), 327 (12), 220 (5), 205 (5), 193 (85), 166 (62), 137 (56), 123 (100), 109 (53), 97 (64), 81 (56), 69 (72), 55 (39).

The other major component, with retention time 16.21 min, gave EIMS peaks at $^{m}/_{z}$ 358 (16, $M^+$) 343 (9), 327 (9), 245 (6), 220 (14), 205 (9), 193 (28), 166 (72), 137 (33), 123 (100), 109 (43), 95 (39), 81 (39), 69 (59), 55 (33). The minor component with retention time 16.86 min gave EIMS peaks at $^{m}/_{z}$ 372 (12.5M), 341 (100), 217 (33) 205 (21), 189 (11), 165 (24), 149 (9), 137 (43), 123 (11), 95 (21), 91 (10), 81 (21), 77 (7), 96 (28), 55 (18).

**Properties of isozonaroic acid**

The less polar of the two compounds, separated by HPLC of the mixture obtained from silica gel column chromatography of Fraction 4 (0.377g, 0.0073% based on wet weight alga), isozonaroic acid (11)

mp 107-108°; $[\alpha]^{25}_{D}$ + 14.92° (c 1.14 in $CHCl_3$);
UV (MeOH) $\lambda_{max}$252nm (ε8300);
IR (KBr) 3600-3300, 2920, 1690, 1280, 780 $cm^{-1}$;
$^1$H NMR ($CDCl_3$) δ7.84 (dd, 1H, J = 8.3, 1.95), 7.80 (dd, 1h, J = 8.1, 1.76), 6.78 (d, 1H, J = 8.37), 5.40 (br s, 1H), 2.67 (d, 2H, J = 6.08) 2.60-1.50 (saturated methylenes), 1.43 (s, 3H), 0.915 (s, 6H, 2 x CH3), 0.89 (s, 3H);
$^{13}$C NMR ($CDCl_3$)δ (multiplicities) 171.65 (s), 158.21 (s), 135.03 (s), 132.42 (d) 130.11 (s), 129.41 (d), 133.68 (d), 121.60 (s), 115.15 (d), 53.81(d), 50.17 (d), 42.18 (t) 39.56 (t), 36.95 (s), 33.24 (q), 33.07 (s), 25.99 (s), 23,79 (t), 22.38 (q), 18.95 (t) 13.93 (q);
EIMS $^m/_z$ 343 (3), 342 (11), 372 (6), 257 (2), 231 (3), 109 (100), 107 (22), 105 (13), 97 (13), 95 (49), 81 (26), 77 (15), 69 (40), 119 (13), 109 (100), 107 (22), 105 (13), 97 (13), 95 (49), 81 (26), 77 (15), 69 (40), 67 (19), 59 (22), 57 (29), 55 (37), 45 (12), 44 (21), 43 (29), 41 (45), 31 (26), 41 (45), 31 (26), 29 (28).
HREIMS: 342.2189 $C_{22}H_{30}O_3$), Calcd. 342,2195.

**Isozonaroic Acid Methyl Ester**

To 50mg of isozonaroic acid in chloroform were added a few drops of diazomethane at room temperature and the solution was allowed to stand for 15 min. Capillary GC of this product on an SP 2100 column (10m long, with split injection ratio 10:1, helium flow rate 2-mL/min, head pressured 10 psi, chart speed 76cm/hr) gave a peak with retention time 12.91 min
EIMS data: $^m/_z$ 356 (23, M), 341 (8), 325 (9), 232 (31), 217 (58), 191 (100), 173 (23), 165 (55), 149 (8), 135 (23), 121 (35), 109 (98), 95 (53), 91 (18), 81 (21) 77 (13), 69 (36), 55 (30), 41 (31).

**Hydrogenation of Isozonaroic Acid**

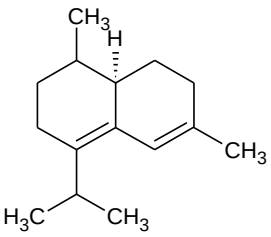
Isozonaroic acid (3 mg) was dissolved in methanol (5 mL) in a round-bottomed flask, platinum oxide (5 mg) was added and hydrogen was passed through the reaction mixture for 5 hours, after which the solution was filtered and solvent was removed under reduced pressure to give the product in quantitative yield; FABMS $^m/_z$ 345 (M+H). HPLC of the reduction product of isozonaroic acid was run under the same conditions as that of the reduction product of zonaroic acid. Two peaks with retention times of 26.8 min and 29.8 min were observed.

**Dihydroisozonaroic acid methyl ester**

To 100µg of the reduction product of isozonaroic acid in chloroform were added a few drops of diazomethane at room temperature and the solution was allowed to stand for 10 min. GC/MS of the ester employing capillary GC(10m long, SP 2100 column, split injection ratio 10:1, helium flow rate 2mL/min, head pressure 10 psi, chart speed 76cm/hr) showed two major peaks with retention times 15.89 min and 16.23 min. The major component with retention time 15.89 min gave EIMS data peaks at $^m/_z$ 358.(10, $M^+$) 343 (5), 327 (13), 220 (5), 205 (5), 193 (84), 165 (60), 137 (58, 123 (100), 109 (56), 97 (63), 81 (41), 69 (73), 55 (42), while the other major components gave EIMS peaks at $^m/_z$ 358 (17), 343 (11), 327 (10), 220 (14), 205 (10), 193 (29), 179 (7), 166 (73), 137 (33), 123 (100), 109 (43), 97 (42), 81 (39), 69 (57), 55 (35). The minor component with retention time 16.68 min gave EIMS peaks at $^m/_z$ 372 (7, $M^+$) 365 (6), 341 (10), 233 (6), 218 (21), 203 (14), 191 (79), 177 (21), 165 (100), 147 (7), 137 (32), 123 (32), 109 (38), 95 (37), 91 (16), 81 (30), 77 (9), 69 (44), 55 (31), while the other minor components gave EIMS peaks at $^m/_z$ ratio 378 (90, $M^+$) 354 (28), 341 (22), 325 (17), 257 (4), 243 (4), 230 (51), 217 (24), 207 (77), 189 (41), 173 (18), 165 (82), 157 (10), 145 (15), 133 (20)|, 123 (100), 109 (31), 95 (32), 91 (18), 81 (29), 77 (13), 69 (57), 55 (39).

GC and GC/MS were also performed on the mixed esters of the reduction product of zonaroic acid and isozonaroic acid under the same conditions as for the separate esters. Two major peaks with retention times 15.94 min and 16.32 min and two minor peaks (4% of the major peaks) with retention times 16.71 min and 17.92 min were observed. The major component with retention time 15.94 min gave EIMS peaks at $^m/_z$ 358 (11, $M^+$) 327 (12), 220 (4), 205 (4), 193 (88), 166 (63), 137 (59), 123v (100), 109 (55), 97 (63), 81 (44), 777 (8), 69 (71), 55 (38), while the other major components gave EIMS peaks at $^m/_z$ 358 (18, M+), 343 (9), 327 (9), 220 (13), 205 (9), 193 (30), 166 (74), 137 (35), 123 (100), 109 (44), 97 (41), 81 (38), 77 (5), 69 (57), 55 (33). The minor components with retention time 16.71 min gave EIMS peaks at $^m/_z$ 372 (4, M), 356 (4), 341 (15), 327 (8), 218 (20), 205 (17), 191 (67), 177 (21), 165 (100), 147 (80), 137 (25), 123 (42), 109 (42), 95 (41), 81 (29), 77 (10), 69 (50), 55 (34), while the other gave EIMS peaks at $^m/_z$ 378 (8, $M^+$), 341 (100), 217 (34), 205 (19), 189 (11), 165 (26), 149 (10), 137 (43), 123 (12), 109 (11), 95 (22), 81 (24), 77 (6), 69 (30), 55 (21).

**Chemical structures**

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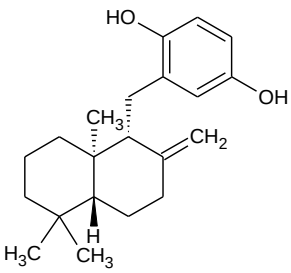


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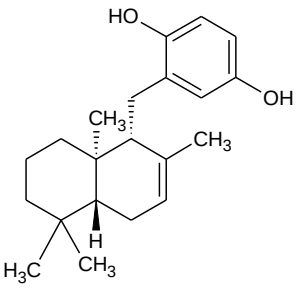


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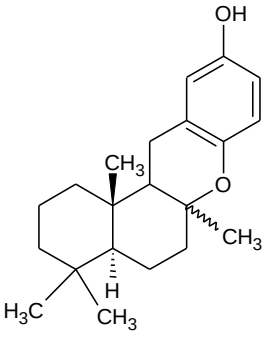


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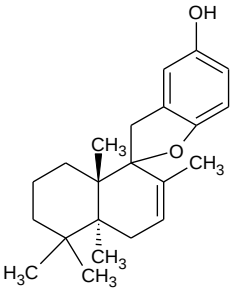


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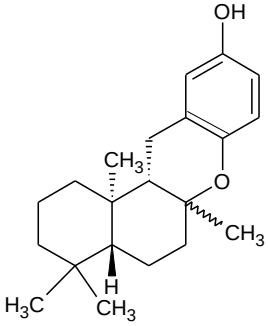


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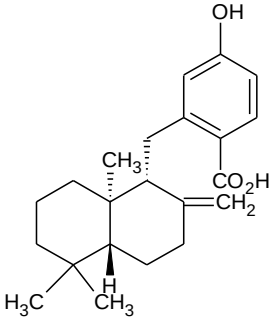


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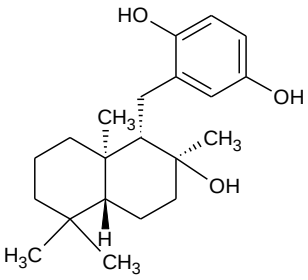


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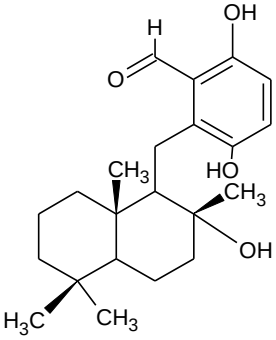


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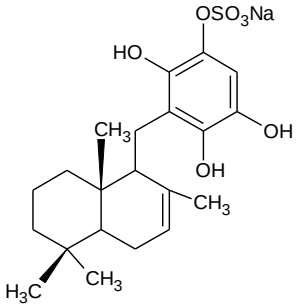


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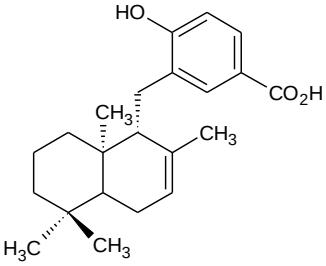


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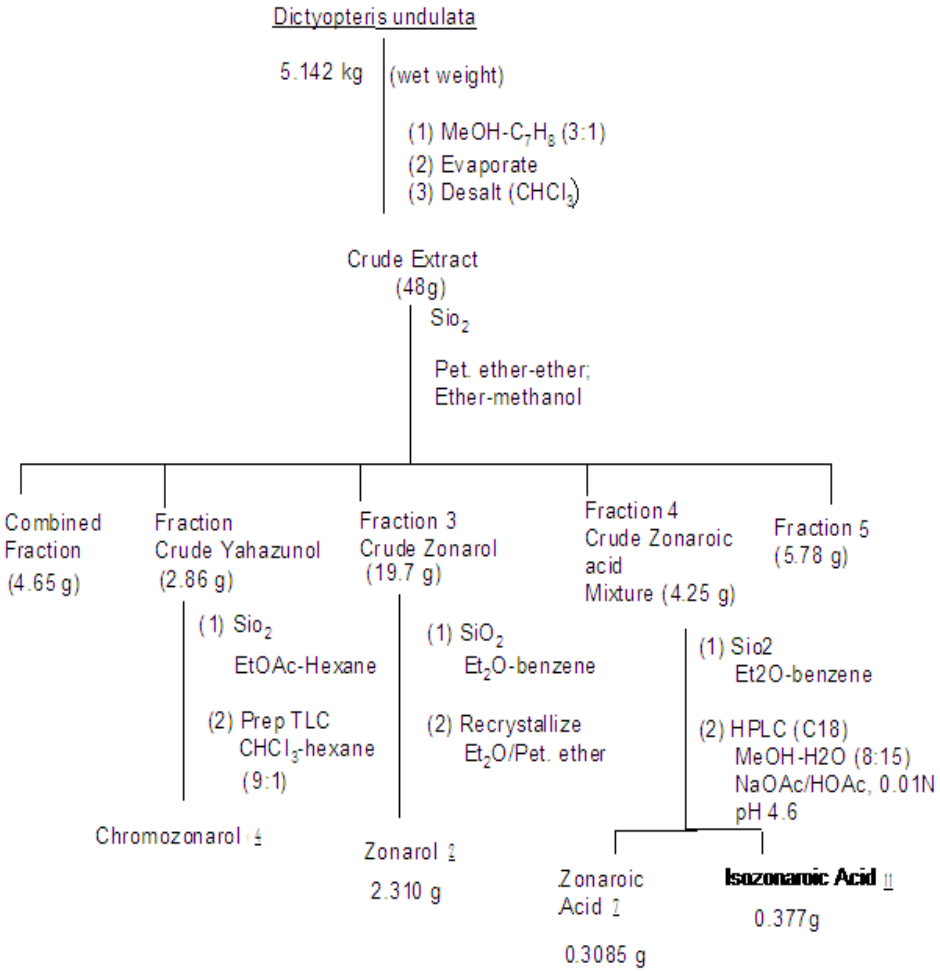


**Scheme 1**

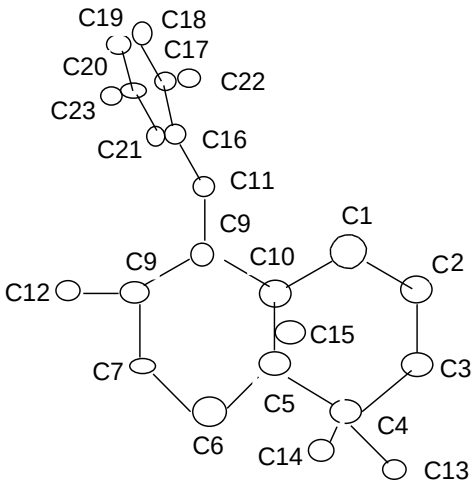


**Figure 1** Perspective drawing of X-ray structure of zonarol 2

**Table I:** NMR signals of zonarol 2

| | $^1$H, 360 MHz ($CDCl_3$) | | | $^{13}$C, 25 MHz ($CD_3COCD_3$) | |
|---|---|---|---|---|---|
| Position | $\delta$, ppm | mult[a] | J (Hz) | $\delta$, ppm | mult[a] |
| 1 | | m | | 38.97 | t |
| 2 | 1.0-2.0 | | | 20.04 | t |
| 3 | | | | 39.68 | t |
| 4 | | | | 34.08 | s |
| 5 | 1.0-2.0 | | | 56.26 | d |
| 6 | | | | 23.90 | t |
| 7 | 2.16 | t | 6.21, 5.84 | 42.81 | t |
| 8 | | | | 129.86 | s |
| 9 | 2.4 | d | 1.48 | 56.66 | d |
| 10 | | | | 40.40 | s |
| 11 | 2.70 | d | 6.32 | 25.03 | t |
| 1 ‘ | | | | 148.33 | s |
| 2’-OH | 4.40b | s | | 148.69 | s |
| 3’ | 6.62 | d | 8.02 | 116.76 | d |
| 4’ | 6.50b | d,d | 8.53, 2.52 | 115.76 | d |
| 5’-OH | 4.54 | s | | 150.60 | s |
| 6’ | 6.59 | d | 3.91 | 112.85 | d |
| 12 | 4.81, 4.68 | s,s | | 108.05 | d |
| 13 | 0.81b | s | | 33.95 | q |
| 14 | 0.83b | s | | 22.06 | q |
| 15 | 0.89 | s | | 14.94 | q |

[a] multiplicity: s= singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Multiplicity for $^{13}$C NMR refers to the off-resonance decoupled spectrum. [b] values could be interchanged

**Table II:** NMR Signals of Chromazonarol 4

| | $^1$H, 220 MHz ($CDCl_3$) | | | $^{13}$C, 25 MHz ($CD_3COCD_3$) | |
|---|---|---|---|---|---|
| Position | $\delta$, ppm | mult[b] | J(Hz) | $\delta$,ppm | mult[b] |
| 1 | | | | 39.69 | t |
| 2 | 0.9-1.0, 1.2-1.8 | m | | 19.05 | t |
| 3 | -- | | | 41.61 | t |
| 4 | -- | | | 33.72 | s |
| 5 | | | | 52.58 | d |
| 6 | 0.9-1.0, 1.2-2.0 | m | | 20.28 | t |
| 7 | | | | 42.34 | t |
| 8 | -- | d | | 77.36 | s |
| 9 | 2.05 | d | 3.20 | 56.20 | d |
| 10 | -- | | | 37.31 | s |
| 11 | 2.58 | d | 9.09 | 23.02 | t |
| 1’ | -- | d | | 123.82 | s |
| 2’ | -- | d | | 149.17 | s |
| 3’ | 6.64 | d,d | 7.50, 0.93 | 118.00 | d |
| 4’ | 6.57 | d,d | 7.56, 2.75 | 114.80 | d |
| 5’- OH | 4.46 | s | | 47.47 | s |
| 6’ | 6.61 | d | 1.44 | 116.39 | d |
| 12 | 1.17 | s | | 21.22 | q |
| 13 | 0.84+ | s | | 33.97 | q |
| 14 | 0.88+ | s | | 22.15 | q |
| 15 | 0.90+ | s | | 15.37 | q |

[a] values marked with same superscripts could be interchanged [b] multiplicity: s= singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Multiplicity for $^{13}$C NMR refers to the off-resonance decoupled spectrum.

**Table III:** NMR signals of zonaroic acid 7

| | $^1$H, 220 MHz ($CDCl_3$) | | | $^{13}$C, 25 MHz ($CD_3COCD_3$) | |
|---|---|---|---|---|---|
| Position | $\delta$ , ppm | mult[a] | J(Hz) | $\delta$ ,ppm | mult[b] |
| 1 | 1.0-2.0 | m | | 39.15 | t |
| 2 | | | | 19.42 | t |
| 3 | | | | 42.10 | t |
| 4 | | | | 33.62 | s |
| 5 | | | | 55.56 | d |
| 6 | 1.0-2.0 | m | | 24.40 | t |
| 7 | | | | 38.12 | t |
| 8 | | | | 121.48 | s |
| 9 | | | 5.02 | 55.84 | d |
| 10 | | | | 40.21 | s |
| 11 | 2.78 | d | | 23.46 | t |
| 1’ | | | | 148.75 | s |
| 2’- OH | | | | 158.66 | s |
| 3’ | 6.78 | d | | 115.10 | d |
| 4’ | 7.78 | dd | 7.32, 1.62 | 132.40 | d |
| 5’ | | | | 128.57 | d |
| 6’ | 7.83 | d | 1.62 | 129.57 | d |
| 7’ | | | | 171.79 | s |
| 12 | 4.82, 4.68 | s, s | | 107.55 | t |
| 13 | 0.82b’ | | | 33.62 | q |
| 14 | 0.84b | | | 21.74 | q |
| 15 | 0.94b | | | 14.52 | q |

[a] multiplicity: s= singlet, d = doublet, t = triplet, q = quartet m = multiplet.
Multiplicity for 13C NMR refers to the off-resonance decoupled spectrum. [b] Values could be interchanged

**Table IV:** NMR signals of isozonaroic acid 11

| | $^1$H, 220 MHz ($CDCl_3$) | | | $^{13}$C, 25 MHz ($CD_3COCD_3$) | |
|---|---|---|---|---|---|
| Position | $\delta$ , ppm | mult[a] | J(Hz) | $\delta$ ,ppm | mult[b] |
| 1 | | | | 39.56 | t |
| 2 | 0.9-1.4; 1.5-2.0 | m | | 18.95 | t |
| 3 | | | | 42.18 | t |
| 4 | | | | 33.07 | s |
| 5 | 0.9-1.4; 1.5-2.0 | m | | 50.17 | d |
| 6 | 2.04 | d,d | 13.6 | 25.99 | t |
| 7 | 5.40 | br s | | 115.18 | d |
| 8 | | | | 121.60 | s |
| 9 | 2.50 | br s | | 53.81 | d |
| 10 | | | | 36.96 | d |
| 11 | 2.65 | d | 5.55 | 23.79 | t |
| 1’ | | | | 135.03 | s |
| 2’-OH | | | | 158.21 | s |
| 3’ | 6.79 | d | 8.40 | 129.41 | d |
| 4’ | 7.80 | dd | 8.48 | 122.68 | d |
| 5’ | | | | 130.11 | s |
| 6’ | 8.02 | d | 1.46 | 132.42 | d |
| 7’ | | | | 171.65 | s |
| 12 | 1.43 | s | | 22.38 | q |
| 13 | 0.89b | s | | 33.24 | q |
| 14 | 0.90b | s | | 21.98 | q |
| 15 | 0.91b | s | | 13.93 | q |

[a] multiplicity: s= singlet, d = doublet, t = triplet, q = quartet m = multiplet.

Multiplicity for 13C NMR refers to the off-resonance decoupled spectrum. [b] Values could be interchanged

**Table V:** Biological activities of zonarol (2), chromazonarol (4), zonaroic acid (7) and isozonaroic acid (11)

| | | zone of inhibition (mm) | | | |
|---|---|---|---|---|---|
| | Organism | 2 | 4 | 7 | 11 |
| Gram –positive bacteria | *Bacillus subtillis* | 19 | 19 | 25 | 24 |
| | *Staphylococcus aureus* | 14 | 0 | | |
| | *Sarcina lutea* | 15 | Trace | | |
| | *Mycobacterium avium* | 14 | 17 | | |
| | *Streptococcus pyogenes* | | 15 | | |
| | *Clostridium perfringens* | 18 | 0 | | |
| Gram- negative bacteria | *Escherichia coli* | 0 | 0 | | |
| | *Klebsiella pneumoniae* | 0 | 0 | | |
| | *Solmonella schottmulleri* | | 0 | | |
| | *Proteus vulgaris* | 0 | 0 | | |
| | *Pseudomonas aeruginosa* | 0 | 0 | | |
| | *Bacteroides fragilis* | 26 | 16 | | |
| Fungi | *Penicillinium attrovenatum* | 0 | 0 | 25 hazy | 25 hazy |
| | *Penicillium oxallicum* | Trace | 0 | | |
| | *Saccaharromyces cerevisiae* | 0 | 0 | | |
| | L/1210: ID50 (μg/mL) | | | | |
| | *Tricophyton rubrum* | 6.25 | | | |
| | *Trocophyton violaceum* | 6.25 | | | |
| | *Trichophyton* *Mentagrophytes* UC 4860 | 125 | | | |
| | *Norcadia asteroids* | 125 | | | |
| | *Tricohophyton asterides* | 250 | | | |
| | *Trichophyton* *Mentagrophytes* UC 4797 | 250 | | | |
| | *Cryptococcus neoformans* UC 1139 | 500 | | | |
| | *Cryptococcus* neoforman | 500 | | | |
| | *Sporotrichium schenckii* | 500 | | | |
| | *Microsporium apioperum* | 500 | | | |
| | *Candida albicans* | 500 | | | |
| | *Microsporium canis* | 500 | | | |
| Cytotoxity vs cil cells | (10μL at 3 mg/mL) | 16 mm | | | |
| | In vivo P 388 | inactive | | | |
| Anti-inflammatory, Hind | Paw Edema; (x Aspirin) | 1.6 | | | |
| Antiviral Activity | PR8 | 0/2 | | | |
| | COE | 0/0 | | | |
| | HA-1 | 0/0 | | | |
| | E.R | 0/0 | | | |
| | HSV-1 | 0/0 | | | |
| | HSV-2 | 0/0 | | | |
| | VACC | 0/0 | | | |

[a]PR8 = infleunza virus ; COE – Coxsakie A-21 virus ; HA-1 = parainfleunza-3 virus ; E.r = equine rhinovirus ; HSV-1, HSV-2 = Herpes simplex virus, types 1 and 2 ;
VACC = Vaccinia virus, expressed as cytotoxicity/virus inhibition (1 = 1 – 10, 20 mm zone of inhabitation.

## Discussion

The molecular ion at $^{m}/_{z}$ 314 indicates formula $C_{21}H_{30}O_2$. Proton NMR peaks centred at δ 6.60, 4.50 and $^{13}$C NMR signals at δ 148.3 and 150.6 suggest the presence of a hydroxy benzene ring. The proton NMR doublet at δ 2.7 and $^{13}$C NMR peak at δ 25 indicate a benzylic group. A series of proton multiplets between δ 2.5 and 1.10 indicate the presence of saturated methylene and methine

groups and overlapping singlets at δ 0.85, methyl groups attached to a quaternary carbon. Significant peaks in the mass spectrum at $^{m}/_{z}$ 191, 123 and data already discussed point to the presence of sequiterpenoid and hydroquinone units in these compounds. Proton NMR at δ 4.70, 4.8, and $^{13}$C NMR signals at 108, 129.86, indicate the presence of a terminal methylene group in the sesquiterpenoid moiety of this compound.

A comparison of these along with the IR and UV data with reported work (Fenical *et al.* 1973) indicated that this compound is zonarol 2). Earlier work (Cimino *et al.* 1975) had inferred the stereochemistry of zonarol and zonaroic acid as 5R, 9R, 10S. X-ray crystallography of zonarol obtained from these studies (Fig. 1) confirmed the gross structure of this compound and the relative stereochemistry as has been suggested.

The bioactive compound obtained from the second Combined Fraction gave a high resolution FAB mass spectrum molecular ion (M+H) at $^{m}/_{z}$ 315.2257, $C_{21}H_{31}O_2$ and EI molecular ion at 314, which is the same value as, hence isomeric with zonarol. Peaks at δ 6.64, 6.61, 6.57, 2.58, 118, 116.39, 114.80, and 23.02 in the proton and carbon NMR respectively, indicate the presence of a hydroxy benzene group, while signals at δ 0.90, 0.88, 0.84 and multiplets between 2.0 and 1.17 indicate methyl groups on tertiary carbons in a sesquiterpenoid moiety. This was corroborated by EIMS data with prominent peaks at $^{m}/_{z}$ 191 and 123.

The above data suggested similarity in structure of this compound to zonarol. There were however differences: this compound showed a proton NMR singlet at δ 1.17 assigned to a methyl group on an oxygen-bearing carbon which was corroborated by $^{13}$C NMR signals at δ 21.22 and 77.36 respectively.

A comparison of the chemical shifts and carbon NMR off-resonance multiplicities of C-7, C-8, and C-9 of this compound (Table II) indicated the difference in structure lies at C-8. This coupled with the proton NMR evidence for the singlet at δ 1.117 indicates that C-8 of this compound bears oxygen and methyl groups. The chemical and spectroscopic evidence when compared with published data ((Fenical *et al.*, 1975) indicated that this compound is chromazonarol 4.

Further evidence for the structure of chromazonarol was obtained from chemical transformation and inter-conversion. Zonarol was treated with pTsOH in benzene to yield a product whose proton, carbon NMR, EIMS, optical rotation and $R_f$ value were identical to the natural product whose structure has been assigned as chromazonal 4. The synthetic compound obtained was converted to the acetate ($Ac_2O$/pyridine) and was found to be very similar to the acetate of the natural product prepared under the same conditions, with respect to their proton NMR, FABMS and $R_f$ value.

The more polar compound obtained from Combined Fraction 4 had proton NMR signals at δ 7.83, 7.78, 6.78, 2.78 and $^{13}$C NMR at 129.57, 132.4, 115.10, 23.46 corresponding to the presence of a benzylic group. The IR had a peak at 16000 cm-1 due to the presence of a conjugated carbonyl group which was corroborated by a peak at δ 171.97 in the $^{13}$C NMR, thus indicating the carbonyl group is in the aromatic ring. $^{1}$H NMR peaks at δ 0.94, 0.84, 0.83 (methyls), between δ 2.4 and 1.0 indicate the presence of saturated methylene and methine groups in a sequiterpenoid moiety.

The mass spectrum showed a molecular ion at $^{m}/_{z}$ 342 ($C_{21}H_{30}O_3$) with a base peak at 191 and another prominent peak at 151. The proton NMR peaks at δ 4.82, 4.68, carbon NMR signals at δ 121.48 and 107.55 and the IR peak at 900 cm-1 point to an exocyclic double bond in the sequiterpenoid moiety. The above data are

similar to those reported (Cimino *et al.* 1975) for the structure of zonaroic acid 7.

The less polar of compounds obtained from this fraction with HREIMS $^{m}/_{z}$ 342.2189, ($C_{22}H_{30}O_3$) was found to be isomeric with zonaroic acid. The IR bands between 3600 and 3300, at 1690 cm-1, $^1$H NMR signals at δ 7.80, 6.79 and peak at $^{m}/_{z}$ 151 ($C_8H_7O_3$ from HREIMS) in the mass spectrum point to a hydroxy benzoic acid group, corroborated by $^{13}$C NMR peaks at δ 171.65, 129.41, 122.68.

The two proton doublets at δ 2.65 and $^{13}$C NMR, signal at δ 23.79 indicate that the hydroxybenzoic acid group is benzylic. The IR peaks at 2920, 1280 cm$^{-1}$, $^1$H NMR signals between δ 1.14 and 0.95, singlets between 0.95 and 0.8 major peaks at $^{m}/_{z}$ 191 (M-151) and 109 (base peak) indicate the presence of a sesquiterpenoid moiety to which methyl groups are attached. The IR peak at 780 cm$^{-1}$, a one proton broad NMR singlet at δ 5.4 points to the presence of a trisubstituted double bond in this moiety. This is corroborated by a one proton broad singlet at δ 2.50, a two proton doublet of doublets at δ 2.04, three proton singlet at 1.43 and $^{13}$C NMR signals at 53.81, 25.49 and 22.38 respectively. Irradiation of the allylic signals at δ 2.50, δ 2.04, caused the broad singlet at δ 5.4 to sharpen considerably and signal at δ 115.18 indicate it to be vinylic. The $^{13}$C NMR signal at δ 121.60 indicates the presence of a tetrasubstituted carbon to which is attached the vinyl methyl. On the basis of the foregoing the structure of this compound point to an isomer of zonaroic acid in which the double bond is endocyclic and named isozonaroic acid 11.

Further evidence for the structure assigned to isozonaroic acid was obtained from hydrogenation and related studies of this compound. Hydrogenation of this compound yielded two diastereomeric products whose HPLC peaks had retention times 26.7 and 29.4 min. Treatment of this mixture with diazomethane led to esterification of the carboxylate group in each diastereomer as major products to a lesser extent methylation of the phenolic group in the hydroxybenzoic acid moiety in the compound as minor products. This was evidenced from the capillary GC and GC-MS data: major peaks for each diastereomer: $^{m}/_{z}$ 358 (M+), 372 (M-$OCH_3$), 193 ($C_9H_9O_3$), 165 (M- $C_9H_9O_3$); peaks for minor products from diastereomer $^{m}/_{z}$ 372 (M+), 341 (M-$OCH_3$).

The more polar zonaroic acid on subjection to the same sequence of reactions, HPLC, Capillary GC and GC-MS gave data on products obtained therefrom that were identical to those obtained for the isomeric isozonaroic acid HPLC of the hydrogenation products obtained from zonaroic acid and isozonaroic acid gave two peaks with retention times 21.6 and 22.6 min on co-injection, thus providing additional evidence for the isomeric nature of these two compounds.

*Biological activity.*

Zonarol showed antibacterial activity against both Gram-positive and Gram-negative organisims (See Table IV). The minimum inhibitory concentration (MIC's) for this compound against a number of fungi indicated modest activity against *Trichophyton* species. Zonarol showed appreciable inhibition of L 1210 cell growth, cytotoxicity, and mild antiviral activity against one RNA influenza virus (Table V). It also showed anti-inflammatory activity 1.6 times that of aspirin.

Chromazonarol exhibited moderate activity against Gram-positive bacteria, little activity against Gram-negative bacteria and scarcely any antifungal activity (Table V). It was generally slightly less activity than zonarol.

Zonaroic acid showed high activity against *B. subtilis* (zone of inhibition 25mm) and moderate antifungal activity (zone of

inhibition 25mm, though hazy). It also exhibits good inhibition of L1210 cell growth, the $ID_{50}$ being 0.07μg/mL. It thus was more active than zonarol (Table V).

Isozonaroic acid showed appreciable activity against *B. subtilis* (zone of inhibition 25mm) and moderate antifungal activity (zone of inhibition 25mm, though hazy). In addition it inhibited the growth of L1210 cells at a level slightly better than zonaroic acid; the $ID_{50}$ being 0.065 μg/mL.

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