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STRUCTURE ELUCIDATION OF HELIOSCOPINOLIDES D AND E FROM *EUPHORBIA CALYPTRATA* CELL CULTURES

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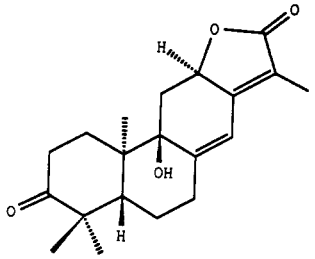
ABSTRACT.—Two novel diterpene lactones **1** and **4**, belonging to the group of helioscopinolides, have been isolated, together with the already known helioscopinolides C [**2**] and A [**3**], from suspension cultures of *Euphorbia calyptrata*, a poisonous plant growing in the Sahara desert. All compounds have been extensively characterized by ms, ^{1}H nmr, and ^{13}C nmr (COSY, NOESY, and HETCOR). Complete nmr assignments, together with the determination of conformation and configuration, are given for **1** and **4**, as well as for the known **2** and **3**. The isolation of all compounds is also described.

In our search for biologically active natural products, we have recently identified four diterpene lactones, active on the central nervous system, in cell cultures of *Euphorbia calyptrata* var. *involucrata* Batt. (Euphorbiaceae) (1). The isolation and the structure determination of these compounds, the novel diterpenes **1** and **4** and the already known **2** and **3**, is herewith reported. Since they have the same carbon skeleton as helioscopinolides A, B, and C, previously isolated from *Euphorbia helioscopia* L. (2), they have been named helioscopinolides D [**1**] and E [**4**]. Compound **4** has already been obtained



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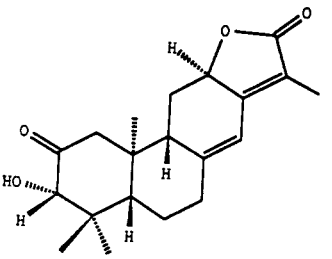

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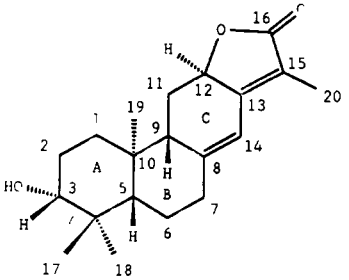

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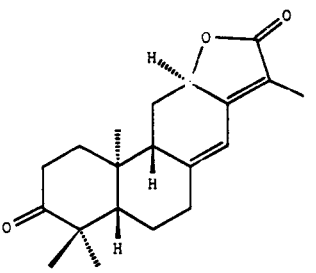

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by chemical oxidation from helioscopinolides A and B (2), but it has never been isolated before from natural sources.

E. calyptrata var. *involucrata* is an uncommon shrub growing in the Sahara desert. A decoction obtained by natives from its roots was commonly said to have some neurotoxic effects. A specimen of the plant was insufficient for isolation and structural determination of the active compounds, and cell cultures were therefore obtained from seeds present in the specimen, in order to have an almost unlimited source of plant material. Si gel cc of an EtOAc extract of *E. calyptrata* cell culture afforded several fractions containing a group of products having analogous uv spectra. The fraction giving the main absorbance was submitted to further purification by preparative tlc and provided four products that were analyzed by ms and nmr.

RESULTS AND DISCUSSION

Because from a preliminary inspection of nmr data the unknown compound **1** was recognized to have the same carbon skeleton as compounds **2–4**, it was decided to perform the main body of spectroscopic work on product **3**, which was obtained in satisfactory amounts and purity, although its crystal structure data had already been published (3).

STRUCTURAL ELUCIDATION OF HELIOSCOPINOLIDE A [**3**].—Compound **3** has a mol wt of 316 daltons and molecular formula $C_{20}H_{28}O_3$. In the ^1H- and ^{13}C-nmr spectra of **3** (Tables 1, 2) a number of characteristic features can be identified: three

TABLE 1. ^1H nmr Data of Compounds **1–4**.[a]

Proton	Compound			
	1	**2**	**3**	**4**
H-1ax . . .	2.38 m	2.39 dd (1.4, 12.4)	1.26 m	1.5–1.7 m
H-1eq . . .	1.85 m	2.74 d (12.4)	1.97 ddd (3.9, 3.9, 13.2)	2.2–2.3 m
H-2ax . . .	2.5–2.6 m	—	1.64 m	2.65 ddd (6.4, 12.6, 15.7)
H-2eq . . .		—	1.77 dddd (3.9, 3.9, 4.3, 13.2)	2.48 ddd (3.6, 5.7, 15.7)
H-3ax . . .	—	3.98 dd (1.4, 5.0)	3.29 dd (4.3, 11.6)	—
H-5ax . . .	2.47 dd (3.3, 12.9)	1.83 dd (2.7, 12.5)	1.17 dd (2.6, 12.6)	1.5–1.7 m
H-6ax . . .	1.53 dddd (4.3, 12.9, 13.1, 13.8)	1.53 dddd (4.1, 12.5, 13.3, 13.5)	1.47 dddd (4.2, 12.6, 13.2, 13.5)	1.5–1.7 m
H-6eq . . .	1.77 m	1.99 m	1.87 dddd (2.6, 2.6, 5.6, 13.2)	1.82 m
H-7ax . . .	2.78 dddd (2.3, 5.3, 13.8, 13.8)	2.31 m	2.21 ddd (5.6, 13.5, 13.5)	2.2–2.3 m
H-7eq . . .	2.35 m	2.61 ddd (2.5, 4.1, 13.7)	2.54 ddd (2.6, 4.2, 13.5)	2.5–2.6 m
H-9ax . . .	—	2.51 d (8.7)	2.18 d (8.6)	2.27 d (8.7)
H-11ax . . .	1.39 dd (13.0, 13.6)	1.62 ddd (8.7, 13.4, 13.7)	1.52 ddd (8.6, 13.5, 13.5)	1.5–1.7 m
H-11eq . . .	3.11 dd (6.2, 13.6)	2.43 dd (6.2, 13.7)	2.56 dd (6.2, 13.5)	2.5–2.6 m
H-12	4.90 ddq (1.8, 6.2, 13.0)	4.85 ddq (1.8, 6.2, 13.4)	4.87 ddq (1.7, 6.2, 13.5)	4.89 ddq (1.7, 6.3, 13.5)
H-14	6.42 d (2.3)	6.37 m	6.29 m	6.35 m
H-17eq . . .	1.16 s	1.24 s	1.05 s	1.14 s
H-18ax . . .	1.08 s	0.73 s	0.84 s	1.07 s
H-19	1.10 s	0.93 s	0.94 s	1.10 s
H-20	1.88 d (1.8)	1.86 d (1.8)	1.84 d (1.7)	1.82 d (1.7)
3-OH . . .	—	3.43 d (5.0)	3.50 s	—
9-OH . . .	3.49 s	—	—	—

[a]Chemical shifts (ppm), multiplicity, and coupling constants (Hz, in parentheses).

TABLE 2. ^{13}C nmr Chemical Shift Data (ppm) of Compounds **1–4**.

Carbon	Compound			
	1	**2**	**3**	**4**
C-1	30.5	51.2	37.4	37.4
C-2	34.2	209.4	27.6	34.4
C-3	216.4	82.3	78.6	215.6
C-4	47.1	45.1	39.1	47.5
C-5	46.0	53.4	54.4	54.8
C-6	24.1	23.0	23.4	24.6
C-7	32.2	36.3	36.9	36.6
C-8	152.2	149.2	151.4	150.2
C-9	76.9	51.3	51.5	50.7
C-10	43.8	46.9	41.2	40.9
C-11	40.0	27.6	27.5	27.8
C-12	76.9	75.3	75.8	75.6
C-13	154.6	155.0	156.0	155.5
C-14	115.9	115.3	114.2	114.8
C-15	118.1	117.6	116.6	117.1
C-16	174.6	174.9	175.3	175.1
C-17	27.1	29.5	28.6	26.5
C-18	21.7	16.4	15.5	21.8
C-19	17.8	17.3	16.6	16.2
C-20	8.3	8.3	8.2	8.3

methyl groups (δ_H 0.84, 0.94, 1.05; δ_C 15.5, 16.6, 28.6) bonded to the two quaternary carbons (δ_C 39.1, 41.2) and one methyl group (δ_H 1.84, δ_C 8.2) bonded to an olefinic carbon; an isolated olefinic hydrogen (δ_H 6.29, δ_C 114.2); a CHOH group (δ_H 3.29, δ_C 78.6, δ_H OH 3.50) whose protons in DMSO-d_6 appear respectively as a multiplet at δ 3.02 and as a doublet at δ 4.40, coupled with each other. In the downfield region of the ^{13}C-nmr spectrum three more olefinic carbons can be found at δ 116.6, 151.4, and 156.0, together with a peak at δ 175.3 characteristic of a lactone carbonyl carbon.

The examination of the COSY and HETCOR spectra allows identification of three more fragments of the structure of **3**: $\overset{5}{C}H\text{-}\overset{6}{C}H_2\text{-}\overset{7}{C}H_2$, $\overset{1}{C}H_2\text{-}\overset{2}{C}H_2\text{-}\overset{3}{C}HOH$, and $\overset{9}{C}H\text{-}\overset{11}{C}H_2\text{-}\overset{12}{C}H$. The task of linking these three fragments to the other structural components and to one another was based on additional data extracted from the long-range HETCOR experiment (complete data reported in Table 3) and from the long-range cross peaks in the COSY spectrum. The correlations in the NOESY spectrum (Table 4) between the protons on C-3, -5, -9, -12 and Me-19 attached to C-10, elucidate the relative configurations of the above five stereocenters in **3**. As cross peaks can be found between the pairs 12/19, 9/5, and 3/5, then H-12 and H-19 must lie on the same side of the molecular plane, and H-3, H-5, and H-9 on the opposite side, as shown in Figure 1, where the absolute stereochemistry of this class of compounds is reported (2,6), together with the scheme of the observed nOe's. The conformations of rings A, B, and C depicted in Figure 1 are supported by consideration of the values of the vicinal coupling constants (Table 1) and the NOESY data as well. The NOESY data also allow a distinction between the two geminal methyl groups at C-4. The structure obtained from X-ray data (3) contained in the Cambridge Structural Database files was examined with the molecular modeling program InsightII (7). A visual comparison with the structure deduced from our nmr data shows a high level of agreement, except for a slight distor-

TABLE 3. $^{13}C/^{1}H$ Observed Correlations in Long-range HETCOR of Compound **3**.[a]

Carbon	Observed correlations
1	19
2	——
3	17, 18
4	5ax, 17, 18
5	1eq, 7eq, 17, 18, 19
6	5ax
7	14
8	9ax
9	14, 19
10	5ax, 9ax, 19
11	7eq
12	9ax, 11ax, 14
13	7eq, 11eq, 14, 20
14	9ax
15	20
16	20
17	18
18	5ax, 17
19	1ax, 5ax, 9ax
20	12

[a]2D experiment parameters: acquisition time 0.05 sec, 2K acquisition points, delays optimized for $J(CH) = 10$ Hz, 260 fids of 236 transients each, data processed on 2048 × 512 data points with a weighting Gaussian function.

TABLE 4. Observed NOESY Cross Peaks for Compound **3**.[a]

Proton	Cross peaks with:				
1ax	1eq	2eq	3ax	9ax	
1eq	1ax	2ax	2eq	11eq	19
2ax	1eq	2eq	18ax	19	
2eq	1ax	1eq	2ax	3ax	
3ax	1ax	2eq	5ax	17eq	
5ax	3ax	6eq	7ax	9ax	
6ax	6eq	7eq	18ax	19	
6eq	5ax	6ax	7ax	7eq	17eq
7ax	5ax	6eq	7eq	9ax	
7eq	6ax	6eq	7ax	14	
9ax	1ax	5ax	7ax	11ax	
11ax	9ax	11eq			
11eq	1eq	11ax	12	13	
12	11eq	19			
14	7eq	20			
17eq	3ax	6eq			
18ax	2ax	6ax			
19	1eq	2ax	6ax	11eq	12
20	14				

[a]2D experiment parameters: acquisition time 0.4 sec, 2K acquisition points, mixing time 600 msec, 216 fids of 64 transients each, data processed on 2K × 2K data points with a weighting Gaussian function.

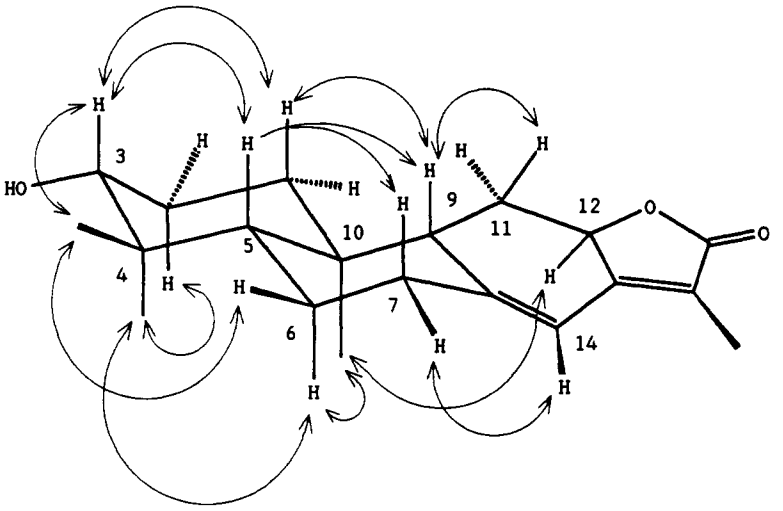

FIGURE 1. Conformation of **3** and main observed nOe's.

tion in ring A, due to steric interaction between Me-18 and Me-19, which is undetectable by nmr analysis.

ELUCIDATION OF THE STRUCTURES OF HELIOSCOPINOLIDES D [**1**], C [**2**], AND E [**4**].—Complete ^{1}H- and ^{13}C-nmr data are reported in Tables 1 and 2.

Compound **2** has a mol wt 14 mass units higher than that of **3** (*m/z* 330) and a molecular formula $C_{20}H_{26}O_4$. The evidence from the COSY experiment and ^{13}C-nmr spectrum suggested a strict similarity with structure **3**, with a carbonyl group in position 2 (δ_C 209.4). A NOESY experiment defined the stereochemistry at C-3, showing that H-3 lies on the same side as H-5.

Compound **4** has a mol wt 2 mass units less than that of **3** (*m/z* 314) and molecular formula $C_{20}H_{26}O_3$. In the ^{1}H-nmr spectrum the disappearance of the 3-OH group is the most evident difference. Also in the ^{13}C-nmr spectrum the C-3 signal is missing at high field, while there is an additional signal at δ 215.6 characteristic of a carbonyl carbon. COSY and HETCOR experiments confirmed the presence of a $\overset{1}{C}H_2$-$\overset{2}{C}H_2$-$\overset{3}{C}O$ fragment in the A ring; furthermore a long-range HETCOR experiment showed correlations of the carbonyl carbon with both the *gem*-dimethyl groups at C-4.

Compound **1** has a mol wt 16 mass units higher than that of **4** (*m/z* 330) and molecular formula $C_{20}H_{26}O_4$. The ^{1}H-nmr spectrum in DMSO-d_6 shows a tertiary hydroxyl group at δ 5.25, and the ^{13}C nmr spectrum exhibits C-9 as a quaternary carbon at δ 76.9, with changes in chemical shifts for the neighboring carbons, as expected for a hydroxyl substitution in position 9. The above evidence suggests that **1** is the 9-hydroxy analogue of **4**. To assess the stereochemistry of the C-9 center, a differential nOe experiment was performed with irradiation of the OH proton: the experiment showed significant nOe on H-1_{ax}, H-5, H-7_{ax}, and H-11_{ax}; thus all these protons must lie on the same side of the molecule.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All nmr spectra were recorded at 27° in $CDCl_3$ (unless otherwise stated in the discussion) on a Varian VXR-400-S spectrometer, operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C and using the standard Varian pulse sequences and processing software for mono- and bidimensional experiments. The average concentration of the samples examined was 20 mg/ml. Field desorption mass spectra were recorded on a Varian Mat 311-A mass spectrometer equipped with a combined FI/FD/EI ion source using benzonitrile-activated emitters. The total potential difference between

the field emitter anode and the cathode was about 9–10 kV. The emitter heating current (EHC) was in the range of 0–12 mA and the source temperature was 120°. Ei mass spectra were recorded on a Finnigan MAT TSQ 70 mass spectrometer at an electron energy of 70 eV using the direct exposure probe technique: the probe heating current was programmed from 30 to 1000 mA at a rate of 1200 mA/min; the source temperature was 150°. Exact mass measurements were performed on a VG Analytical 70-70 EQ spectrometer at a resolution of 10.000 (10% valley), using peak matching method and perfluorokerosene as reference compound. Analytical and preparative tlc were carried out on precoated Si gel G (Merk Kieselgel G254) plates. A Beckman Model 332 solvent delivery system equipped with a photo diode array detector Model 168 and two 110 A pumps, was used for hplc on two Merck Hibar RP8 and RP18 columns. Uv spectra were recorded in MeCN solution on a Bausch & Lomb 2000 spectrophotometer. Reagents analytical grade and solvents analytical and hplc grade (C. Erba) were used.

ISOLATION OF COMPOUNDS.—One hundred Erlenmeyer flasks containing 50 ml each of *E. calyptrata* suspended cultures were prepared as reported elsewhere (1). After 20 days they were harvested, pooled and centrifuged. Cells were suspended in 1 liter of MeOH, homogenized by means of an Ultraturrax, and centrifuged. Extraction was repeated twice, and the pooled MeOH extracts were concentrated under reduced pressure. The supernatant of the cultures (3.5 liters) was reduced to 1 liter under reduced pressure, pooled with the previous cell extract, and extracted with 2 liters × 3 of EtOAc. The organic layer was dried on anhydrous Na_2SO_4, concentrated under reduced pressure, and loaded on a Si gel Si 60 (Merck) column (7 × 30 cm) in CH_2Cl_2-petroleum ether (1:1). The column was eluted with CH_2Cl_2 (1 liter), CH_2Cl_2/0.5% EtOH (2 liters), CH_2Cl_2/20% EtOH (1 liter), EtOH (1 liter). Twenty fractions (250 ml) were collected and pooled according to their tlc behavior to give four pools. Among these pool 2, containing almost 60% of the whole uv absorbance, was further purified by preparative tlc. The following solvent systems were used: (A) Et_2O, (B) CH_2Cl_2-EtOH (99:1), (C) CH_2Cl_2-iPrOH (92:8), (D) Et_2O-petroleum ether (90:10), (E) CH_2Cl_2-EtOH (95:5). Pool 2 after repeated purifications provided four products whose purity was checked by hplc (analytical conditions described elsewhere) (1).

Compound **1**.—Yield 37 mg; exact mass measurement $[M]^+$ *m/z* 330.1894 ($C_{20}H_{26}O_4$ requires 330.1831); λ max 270.0 nm, E_1% 1 cm 615.

Compound **2**.—Yield 9 mg; exact mass measurement $[M]^+$ *m/z* 330.1805 ($C_{20}H_{26}O_4$ requires 330.1831); λ max 273.2 nm, E_1% 1 cm 578.

Compound **3**. Yield 32 mg; exact mass measurement $[M]^+$ *m/z* 316.1967 ($C_{20}H_{28}O_3$ requires 316.2038); λ max 275.0 nm, E_1% 1 cm 628.

Compound **4**.—Yield 68 mg, exact mass measurement $[M]^+$ *m/z* 314.1903 ($C_{20}H_{26}O_3$ requires 314.1882); λ max 273.0 nm, E_1% 1 cm 597.

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