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BIOACTIVE ERGOST-5-ENE-3β,7α-DIOL DERIVATIVES FROM *PSEUDOBERSAMA MOSSAMBICENSIS*[1]

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ABSTRACT.—Bioactivity-directed fractionation of the methyl ethyl ketone extract of *Pseudobersama mossambicensis* resulted in the isolation of ergosta-5,24(28)-diene-3β,7α-diol [**1**], 24,28-epoxyergost-5-ene-3β,7α-diol [**2**], and ergost-5-ene-3β,7α,24,28-tetraol [**3**]. All three sterols showed selective activity towards DNA repair-deficient yeast mutants. The sterol **1** also showed cytotoxicity towards wild-type P-388 murine leukemia cells. The isolation, structural elucidation, and biological activities of these sterols are reported. The sterol **3** is most probably an artifact formed from **2** during the isolation process.

The search for new anticancer agents from natural sources has focused in the past largely on extracts that show cytotoxicity to one or more cell lines in vitro. This approach has been quite fruitful, leading to the isolation of taxol among other compounds (1), but it has the limitation that cytotoxicity is a rather unselective screen. In recent years there has thus been an increased emphasis on the discovery of selective agents by means of improved screening methods. The disease-oriented panel of human tumor cell lines currently used by the National Cancer Institute (2) is one method that has been used successfully to identify novel bioactive leads, but this requires a major investment in personnel and facilities and is thus beyond the reach of the average academic laboratory. The use of mechanism-based screens is a second approach to drug discovery, and we have applied this approach in our work.

The screen that we use is based on the differential response of DNA repair-deficient and repair-proficient yeast strains to the test sample. The use of microbiological assays for the detection of potential anticancer activity has been described on several occasions (3–6); such assays have the advantage of speed and simplicity. Yeast screens in particular are desirable because of their closer genetic and biochemical resemblance (eukaryotic vs. prokaryotic) than bacterial screens to mammalian cells.

Three major DNA repair pathways have been defined in yeast; they are known as the RAD3, RAD6, and RAD52 pathways. The RAD3 pathway is associated with excision repair, the RAD6 pathway is the error-prone pathway, and RAD52 is the recombinational pathway associated with repair of double-strand breaks and meiotic recombination (7). Yeasts deficient in each of these repair pathways and also having increased cell membrane permeability have been used to screen for anticancer agents (6) and are available in our laboratory. In the present study only the RAD6 and RAD52 mutants were used.

The assay is carried out by measuring the growth inhibiton of repair-deficient yeasts in comparison with a wild-type yeast having the same permeability mutation. A mutant lacking one of the repair pathways will be more sensitive than the wild-type yeast to DNA damage repaired predominantly by that pathway, and thus agents which cause

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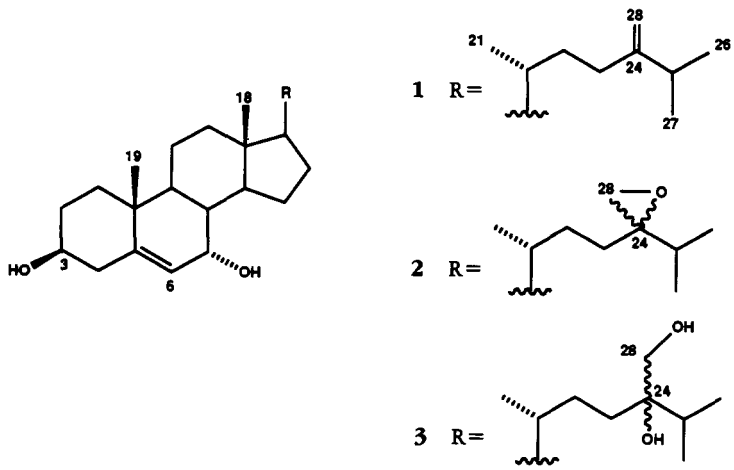
DNA damage can be selectively detected. In our laboratory we report results as IC_{12} values, where IC_{12} is the concentration (in μg/ml) required to produce an inhibition zone of 12 mm diameter around a 100 μl well in the yeast strain in question. An extract is considered active if it shows selective activity against one or more repair-deficient yeasts (IC_{12} less than one-third that of the wild-type yeast) and has an IC_{12} less than 2000.

In the course of a screening program using this assay conducted as part of a National Cooperative Drug Discovery Group in collaboration with the University of Virginia and SmithKline Beecham Pharmaceuticals, a methyl ethyl ketone (MEK) extract of the twigs and leaves of *Pseudobersama mossambicensis* (Sim) Verdc. (Meliaceae) showed selective activity against the RAD52 yeast strain. The only previously reported work on this genus has been the analysis of seeds for their fatty acid composition (8). We thus carried out a bioactivity-directed fractionation of this extract, and we report the isolation, characterization, and biological activity of the three bioactive sterols **1**–**3**, of which **2** and **3** are new.



RESULTS AND DISCUSSION

An initial solvent-solvent partition of the MEK extract of *P. mossambicensis* (prepared by soaking the twigs and leaves of *P. mossambicensis* with cold hexanes followed by MEK and evaporation of the latter) with hexane/80% aqueous MeOH resulted in enhanced activity in the 80% aqueous MeOH fraction. Flash chromatography of this bioactive fraction on Si gel with a step gradient of $CHCl_3$ and iPrOH gave several bioactive fractions. These were further purified by Si gel preparative tlc, reversed-phase tlc, and reversed-phase hplc, yielding three bioactive compounds. Two of the compounds were obtained as crystalline solids, whereas the third one resisted crystallization. All three compounds gave positive responses to the Liebermann-Burchard test for sterols and had several features in their ^{1}H-nmr spectra (Table 1) and mass spectra (fragments at *m/z* 271 and 253) suggesting a common sterol nucleus; the differences were therefore attributed to their side-chain structures.

Hrms analysis of the least polar sterol **1** gave the molecular formula $C_{28}H_{46}O_2$. Successive losses of two H_2O units from the molecular ion suggested the presence of two OH groups, one of which was located at C-3 on biogenetic reasoning and the other at C-7 based on ^{1}H-nmr spectrum which had a 1H multiplet at δ 3.85 due to H-7α (9,10). The ^{1}H-nmr spectrum also indicated the presence of a vinyl proton on a vinylidene

TABLE 1. Selected ^{1}H-nmr Chemical Shifts (400 MHz) of Sterols **1–3** in $CDCl_3$.[a]

Proton	Compound		
	1	**2**	**3**
H-3	3.60 m ($w_{1/2}$ = 20.0)	3.60 m ($w_{1/2}$ = 20.0)	3.60 m ($w_{1/2}$ = 20.0)
H-6	5.61 d (5.0)	5.62 d (5.0)	5.60 d (5.0)
H-7	3.85 m ($w_{1/2}$ = 11.0)	3.87 m ($w_{1/2}$ = 11.0)	3.85 m ($w_{1/2}$ = 11.0)
H_3-18	0.70 s	0.69 s	0.70 s
H_3-19	1.10 s	1.00 s	0.99 s
H-21	0.97 d (6.5)	0.97 d (6.5)	0.95 d (6.5)
H_3-26	1.25 d	0.91 d	0.91 d
H_3-27	1.35 d (6.2)	0.94 d (6.2)	0.93 d (6.2)
H_2-28	4.65 s, 4.75 s	2.53 d, 2.60 d (6.0)	3.46 m, 3.60 m

[a]Chemical shifts (relative to TMS) are in ppm and coupling constants (in parentheses) in Hz.

group, two methyl groups on quaternary carbons, and three methyl groups on tertiary carbons. The structure of this sterol was elucidated as ergosta-5,24(28)-diene-3β,7α-diol [**1**] with the aid of ^{1}H- and ^{13}C-nmr spectra. The former spectrum was completely analyzed by the application of the ^{1}H-^{1}H COSY technique, and ^{1}H- and ^{13}C-nmr spectral assignments of **1** are given in Tables 1 and 2, respectively. The assignments of carbons C-20 to C-28 were based on analogy to the known values for 24-methylene sterol derivatives (11–13). Simultaneously with our work a paper describing the isolation of **1** from another Meliaceous plant was published by Garcia *et al.* (14). Comparison (tlc, reversed-phase hplc, ^{1}H nmr) of our sterol with an authentic sample confirmed its identity as **1**.

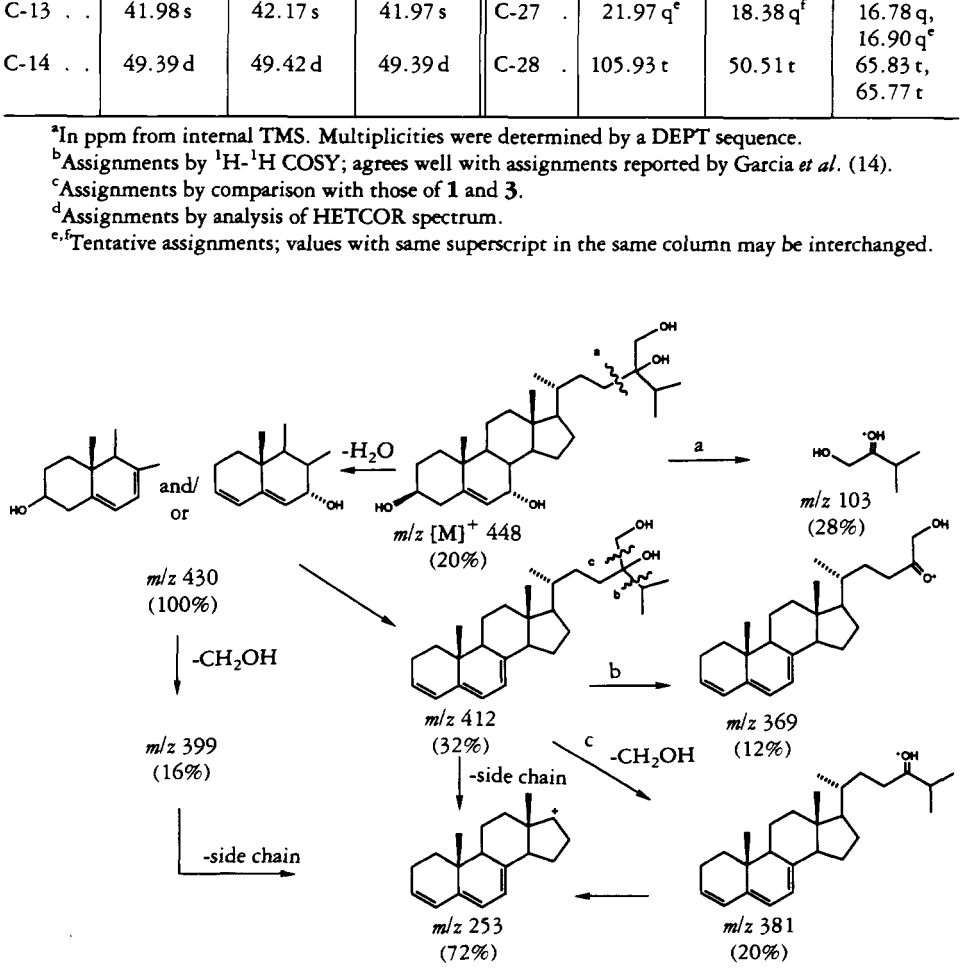
As noted above, the remaining two sterols differed from **1** only in their side-chain structures. The major differences were observed in the ^{1}H-nmr chemical shifts of H_3-26, H_3-27, and H_2-28 (Table 1). In their hrms, the molecular ion peaks of the sterols **2** and **3** showed shifts of 16 and 34 mass units, respectively, towards higher mass, suggesting that **2** may be the 24,28-epoxide of **1** and **3** its diol. The oxirane structure **2** proposed for the second sterol was supported by the presence of two doublets (J = 5 Hz) at 2.53 and 2.60 ppm due to H_2-28 in the ^{1}H-nmr spectrum, and resonances for secondary and quaternary carbons at 50.51 and 62.80 ppm due to C-28 and C-24, respectively, in its ^{13}C-nmr spectrum. These data indicated that **2** is a single enantiomer, but a lack of material prevented us from determining the stereochemistry at C-24.

The third and the most polar sterol **3** had a molecular composition of $C_{28}H_{48}O_4$ and showed a prominent ion in its mass spectrum at *m/z* 430 $[M - H_2O]^+$. The mass spectrum also showed significant peaks at *m/z* 412 $[M - 2H_2O]^+$, 399 $[M - H_2O - CH_2OH]^+$, 381 $[M - 2H_2O - CH_2OH]^+$, 271 $[M - H_2O$ − side chain$]^+$, and 253 $[M - 2H_2O$ − side chain$]^+$. Probable structures of these and other significant fragments are depicted in Scheme 1. The 400 MHz ^{1}H-nmr spectrum of **3** in $CDCl_3$ showed, in addition to the presence of H-3α, H-7β, and H-5, the presence of a CH_2OH group in a chiral environment. This and the remaining OH group were located at C-24 based on the ms frag-

TABLE 2. ^{13}C-nmr Chemical Shifts (100.57 MHz) of Sterols **1–3** in $CDCl_3$.[a]

Carbon	Sterol			Carbon	Sterol		
	1[b]	**2**[c]	**3**[d]		**1**[b]	**2**[c]	**3**[d]
C-1 . .	36.98 t	37.02 t	36.97 t	C-15 .	24.26 t	24.27 t	24.25 t
C-2 . .	31.34 t	31.38 t	31.33 t	C-16 .	28.23 t	28.24 t	28.25 t
C-3 . .	71.34 d	71.36 d	71.31 d	C-17 .	55.62 d	55.52 d	55.40 d, 55.37 d
C-4 . .	42.15 t	42.01 t	41.96 t	C-18 .	11.62 q	11.65 q	11.62 q
C-5 . .	146.21 s	146.25 s	146.21 s	C-19 .	18.22 q	18.25 q	18.22 q
C-6 . .	123.84 d	123.87 d	123.82 d	C-20 .	35.69 d	35.76 d	36.15 d
C-7 . .	65.33 t	65.34 t	65.30 t	C-21 .	18.71 q	18.68 q	18.73 q
C-8 . .	37.37 d	37.41 d	37.47 d	C-22 .	34.62 t	30.31 t[e]	29.82 t, 29.70 t
C-9 . .	42.23 d	42.26 d	42.20 d	C-23 .	30.78 t	27.95 t[e]	28.91 t, 28.89 t
C-10 . .	37.49 s	37.41 s	37.36 s	C-24 .	156.86 s	62.80 s	76.12 s, 76.10 s
C-11 . .	20.68 t	20.71 t	20.67 t	C-25 .	33.77 d	31.68 d	32.63 d
C-12 . .	39.15 t	39.17 t	39.12 t	C-26 .	21.84 q[e]	17.70 q[f]	16.99 q, 17.07 q[e]
C-13 . .	41.98 s	42.17 s	41.97 s	C-27 .	21.97 q[e]	18.38 q[f]	16.78 q, 16.90 q[e]
C-14 . .	49.39 d	49.42 d	49.39 d	C-28 .	105.93 t	50.51 t	65.83 t, 65.77 t

[a]In ppm from internal TMS. Multiplicities were determined by a DEPT sequence.
[b]Assignments by ^{1}H-^{1}H COSY; agrees well with assignments reported by Garcia *et al.* (14).
[c]Assignments by comparison with those of **1** and **3**.
[d]Assignments by analysis of HETCOR spectrum.
[e,f]Tentative assignments; values with same superscript in the same column may be interchanged.



SCHEME 1. Ms fragmentation of **3**.

mentation (Scheme 1) and relationship to the structures of the other two sterols. The assignment was supported by analysis of ^{1}H- and ^{13}C-nmr spectra by ^{1}H-^{1}H COSY, DEPT, and HETCOR techniques and comparison with those of the sterol **1** (see Tables 1 and 2, respectively).

A careful analysis of the expanded ^{13}C-nmr spectrum of **3** indicated that the signals due to C-17, C-22, C-23, C-24, C-26, C-27, and C-28 appeared as doublets with chemical shift differences ranging from 0.12 to 0.02 ppm (Table 2), whereas all the other signals were sharp singlets. This suggested that the compound is a mixture of diastereomers differing in stereochemistry at C-24, and this was supported by additional signals observed for H_2-28 (CH_2OH) in the ^{1}H-nmr spectrum of **3**. The occurrence of **3** as a diastereoisomeric mixture suggests that it may be an artifact arising from **2** during the isolation process, probably on chromatography.

The biological activity data for sterols **1–3** in our mechanism-based yeast mutant bioassays are given in Table 3. All three sterols showed selective activity in the RAD52 assay compared with the wild-type RAD$^+$ strain. Sterol **1** was also tested in P-388 murine lymphocytic leukemia assay and was found to be moderately active. It is noteworthy that cholest-5-ene-3β,7α-diol derivatives are reported to be inactive in some cytotoxicity assays, whereas their 7β counterparts are active (15). We are currently engaged in the synthesis of **1–3** and several of their analogues to determine their structure-activity relationships and for a comparative study of our yeast-based bioassay with some cytotoxicity assays.

TABLE 3. Bioactivity Data of Sterols **1–3** and the Standards.[a]

Compound	Organism or Cell Line[b]			
	RS322YK rad52	RS167N rad6	RS188N RAD$^+$	P-388
1	8.0	12.0	>1,500	4.3
2	0.4	6.0	>50	NT[c]
3	1.0	NT[c]	>100	NT[c]
Camptothecin	0.6	8.7	—	—
Streptonigrin	0.4	2.4	—	—

[a]Results are expressed as IC_{12} (RAD52, RAD6, RAD$^+$) (μg/ml) or IC_{50} (P-388) (μM) values.
[b]P-388, wild-type P-388 murine leukemia cells.
[c]Not tested.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mp's were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were taken in $CHCl_3$ solution with a Perkin-Elmer Model 241 polarimeter. The ^{1}H- and ^{13}C-nmr spectra were recorded on a Varian Unity 400 spectrometer at 400 and 100.57 MHz, respectively, with TMS as internal standard. ^{1}H-^{1}H COSY, DEPT, and ^{1}H-^{13}C HETCOR nmr experiments were performed on the same spectrometer, using standard Varian pulse sequences. Mass spectra were taken on a VG 7070 E-HF instrument. Flash chromatography was performed using Si gel Merck G60 (230–400 mesh), preparative tlc with Si gel GF_{254} plates (Analtech, 500 μm, 20 × 20 cm), and reversed-phase preparative tlc with Whatman PLKC18F linear K reversed-phase (1000 μm, 20 × 20 cm) plates. Hplc purifications were carried out on a Waters apparatus equipped with Whatman Partisil 10 ODS-3 column (4.6 mm i.d. × 25 cm) and a refractive index detector.

BIOACTIVE EXTRACT OF *P. MOSSAMBICENSIS*.—The plant material (PR-54919) was collected in September 1990, in Kenya, and a voucher specimen has been deposited at the Herbarium of the National Arboretum, Agricultural Research Service, USDA, Washington, DC. Extraction of 400 g plant material with hexane followed by MEK yielded 10.3 g (2.6%) of MEK extract. The MEK extract was provided by Prof. S.M. Hecht, Department of Chemistry, University of Virginia.

BIOASSAY.—Strains of genetically engineered yeast (*Saccharomyces cerevisiae*) were provided by Mr. Leo Faucette of SmithKline Beecham Pharmaceuticals. The strains RS188N, RS322YK, RS167N were plated on YPD agar (7mm layer) in 9 × 9 or 23 × 23 cm plates. Wells (6 mm diameter) were made in the plates (9 wells in a 9 × 9 cm plate and 49 wells in a 23 × 23 cm plate) with a cork borer, substances were dissolved in DMSO-MeOH (1:1), and a 100-μl sample was placed in each well. Camptothecin was used as a positive control for RS322YK. Streptonigrin was used for RS167N. Plates were read after incubation for 48 h at 30°, and IC_{12} values were determined from dose-response curves using linear regression with log of dose as Y and zone size as X. The P-388 murine lymphocytic leukemia assay was performed at SmithKline Beecham Pharmaceuticals, Philadelphia. The MEK extract of *P. mossambicensis* showed selective activity against the RAD52 yeast strain (RS322YK), with an IC_{12} value of 640 μg/ml.

ISOLATION OF BIOACTIVE STEROLS.—The isolation process was guided throughout by the results of the RAD52 bioassay. The bioactive MEK extract (6.52 g) was partitioned between 80% aqueous MeOH (1.2 liters) and hexane (1 liter). The hexane layer was washed with 80% aqueous MeOH (6 × 300 ml), and the combined aqueous MeOH layer was then washed with hexane (4 × 500 ml). Evaporation gave 3.93 g of the 80% aqueous MeOH and 1.89 g of the hexane fractions. The bioactive 80% aqueous MeOH fraction (1.00 g) was subjected to flash chromatography on Si gel (2.0 × 30.0 cm) and eluted with $CHCl_3$-iPrOH (90:10) (30 × 20 ml fractions) and $CHCl_3$-iPrOH (80:20) (10 × 20 ml fractions). Based on their tlc patterns, similar fractions were combined to obtain a total of 10 fractions. Three of these combined fractions (III, 133 mg; V, 99 mg and VIII, 36 mg) had significant bioactivity. Each of these was further fractionated and purified by preparative tlc [10% iPrOH in *n*-heptane], reversed-phase tlc (10% H_2O in MeOH), and reversed-phase hplc (15% H_2O in MeOH) to give sterols **1** (12 mg), **2** (4 mg), and **3** (9 mg), respectively, from combined fractions III, V, and VIII.

ERGOSTA-5,24(28)-DIENE-3β,7α-DIOL [**1**].—Colorless crystals from EtOAc/heptane: mp 193–195°; $[\alpha]^{20}D$ −85° (*c* = 0.27, $CHCl_3$); eims *m/z* $[M]^+$ 414.3490 ($C_{28}H_{46}O_2$ requires 414.3497), $[M-H_2O]^+$ 396.3387 ($C_{28}H_{44}O$ requires 396.3392), $[M-H_2O-$ side chain$]^+$ 271, $[M-2H_2O-$ side chain$]^+$ 253. For ^1H- and ^{13}C-nmr spectral assignments see Tables 1 and 2, respectively. The compound had properties (tlc, hplc, ^1H-nmr) identical with those of an authentic sample provided by Dr. Ch. Morin (14).

24,28-EPOXYERGOST-5-ENE-3β,7α-DIOL [**2**].—Amorphous solid which resisted crystallization: $[\alpha]^{20}D$ −37° (*c* = 0.27, $CHCl_3$); eims *m/z* $[M]^+$ 430.3526 ($C_{28}H_{46}O_3$ requires 430.3447), $[M-H_2O]^+$ 412.3302 ($C_{28}H_{44}O_2$ requires 412.3341), $[M-H_2O-$ side chain$]^+$ 271, $[M-2H_2O-$ side chain$]^+$ 253. For ^1H- and ^{13}C-nmr spectral data, see Tables 1 and 2, respectively.

ERGOST-5-ENE-3β,7α,24,28-TETRAOL [**3**].—Colorless crystals from EtOAc/hexane: mp 198–200°; $[\alpha]^{20}D$ −55° (*c* = 0.70, $CHCl_3$); eims *m/z* $[M]^+$ 448.3570 ($C_{28}H_{48}O_4$ requires 448.3553), $[M-H_2O]^+$ 430.3411 ($C_{28}H_{46}O_3$ requires 430.3446); for other significant ms fragments and their assignments see Scheme 1; for ^1H- and ^{13}C-nmr spectral data, see Tables 1 and 2, respectively.

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