Antimycobacterial Cycloartanes from *Borrichia frutescens*

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Received July 15, 1996[⊗]

In a bioassay-guided search for antimycobacterial compounds from higher plants of the southeastern United States, we have chemically investigated the sea daisy (*Borrichia frutescens*) from coastal marshes of Louisiana for their active constituents. Bioactive chromatographic fractions provided two new triterpenes, (24*R*)-24,25-epoxycycloartan-3-one (**1**) and (23*R*)-3-oxolanosta-8,24-dien-23-ol (**4**), and (3αH,24*R*)-24,25-epoxycycloartan-3-ol (**3a**). Compound **3a** had been previously isolated as a mixture of C-24 epimers. The structures of **1**, **3a**, and **4** were established by spectroscopic methods and chemical transformations, and the molecular structures of **1** and **4** were determined by single-crystal X-ray diffraction. In a radiorespirometric bioassay against *Mycobacterium tuberculosis*, the epoxycycloartanes **1** and **3a** exhibited minimum inhibitory concentrations of 8 μg/mL. In contrast, the lanostadiene-type triterpene **4** showed no significant inhibition at 128 μg/mL, as did the acetate **3b**. Cytotoxicity for Vero cells gave IC_{50} values of 71.8, 39.8, and 103.6 μg/mL for triterpenes **1**, **3a**, and **4**, respectively.

In our continued search for biologically active natural products from higher plants of the southeastern USA, we investigated the aerial parts of the sea daisy, *Borrichia frutescens* (L). DC. This monotypic genus of the family Asteraceae, tribe Heliantheae, is a widely distributed halophyte in the saline and brackish coastal marshes of Louisiana and other neighboring Gulf Coast states. A previous chemical study of *B. frutescens* from Veracruz, Mexico, has afforded the triterpenes stigmastanol, stigmasterol, and oleanolic acid as well as the heliangolide-type sesquiterpene lactone zoapatanolide A.[1,2] We describe below the structures of two new triterpenes, one cycloartanone and one lanostadiene, from the flowers of *B. frutescens* collected near Grand Isle, LA.

Results and Discussion

Crude dichloromethane extracts of the flowers, leaves, and stems of *B. frutescens* were tested by a radiorespirometric method for activity against *Mycobacterium tuberculosis* (H37Rv).[3] The highest level of antimycobacterial activity was found in the flower extract, which was separated into eight fractions by a standard VLC procedure using silica gel with increasing solvent polarity (Table 1). Activities of the eight fractions against *M. tuberculosis*, which are also listed in Table 1, indicated that the nonpolar fractions 2−4 of the crude flower extract exhibited the highest inhibitory activity. At 33 μg/mL, all three fractions showed inhibitions of 95% or higher, while all other fractions gave values near 30% or below. Chemical investigation of fractions 2−4 led to the isolation of two new triterpenes (**1** and **4**) and one known triterpene (**3a**); the structures were elucidated as described below.

Compound 1, $C_{30}H_{48}O_2$, mp 119−122 °C, gave strong IR absorptions at 1708 cm^{-1}, suggesting the presence of ketone group(s). The ^{1}H-NMR spectrum of **1** (Table 2) exhibited seven methyl signals and two upfield mutually coupled one-proton doublets at δ 0.57 and 0.78, which indicated the presence of a cyclopropane methylene group in the molecule. The above ^{1}H-NMR data together with a mass spectral peak at *m/z* 440 suggested a cycloartanone-type triterpene skeleton. The absence of an IR OH absorption in **1** together with the NMR spectral comparison of **1** with the known cycloartanone desoxyprefrutecin B (**2**)[4] strongly suggested that compound **1** differed from **2** only in the absence of a C-16 β-hydroxyl group in **1**. This was supported by the lack of the downfield multiplet (H-16) in **1** which appeared at δ 4.44 in 2.[4] Also, the methyl absorption at δ 1.19 in **2** (C-13 methyl) was shifted upfield in **1** (δ 0.99), suggesting the absence of the deshielding β-hydroxyl group at C-16 in **1**.

The ^{13}C-NMR spectrum of **1** (Table 3) and DEPT experiments confirmed the presence of seven methyls, a ketone (δ 216.5), a cyclopropane methylene (δ 29.5, t), and an epoxide group at C-24−25 (δ 64.7 and 58.3). ^{13}C-NMR assignments were based on DEPT experiments as well as spectral comparison with a previously reported structural analogue, which differed from **1** only in the position of the epoxide function (C-23−C-24) in the cycloartenone side chain and the presence of a β-hydroxyl group at C-16 as in **2**.[5] Differences observed

Table 1. Inhibitory Activity of *B. frutescens* Fractions (Flowers, CH_2Cl_2 Extract) against *M. tuberculosis* (H37Rv)

fraction[a]	% hexane	% EtOAc	% MeOH	percent inhibition 33 μg/mL	percent inhibition 100 μg/mL
1	100			31	23
2	95	5		95	95
3	80	20		97	97
4	50	50		95	96
5	20	80		27	48
6		100		26	58
7		50	50	21	19
8			100	24	19

[a] 100 mL of solvent.

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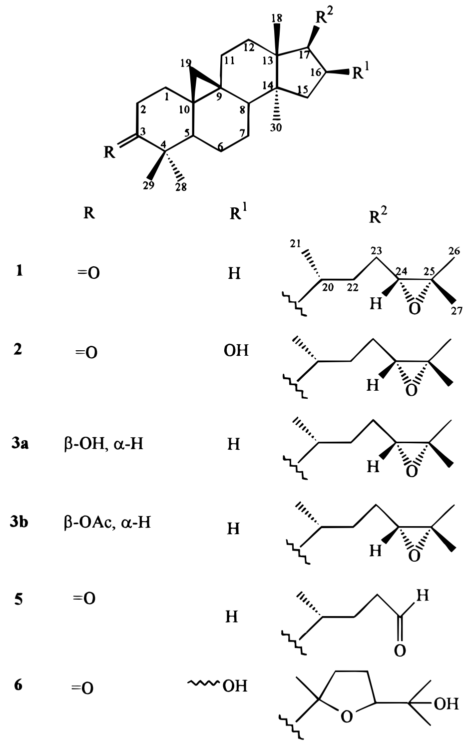
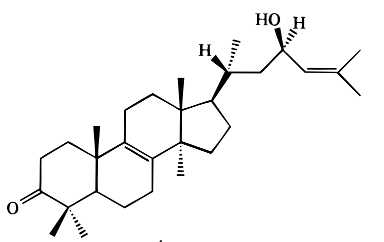
⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.





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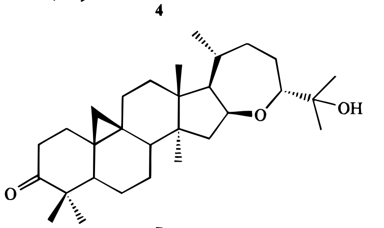

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included the absence of the C-16 oxygen-bearing carbon signal in **1** and the presence of epoxide carbon absorptions in agreement with a C-24−C-25 epoxide group as shown in **1**, exclusive of stereochemistry. The mass spectrum confirmed the molecular weight of **1** with a parent peak *m/z* 440.7 and a strong peak at *m/z* 313 corresponding to the fragment of the tetracyclic ring system by loss of the side chain. Crystallization of **1** from hexane−EtOAc (19:1) provided crystals suitable for single-crystal X-ray diffraction analysis, which unambiguously established the molecular structure and relative stereochemistry of **1** as shown in Figure 1. Table 4 lists its crystallographic data, and the coordinates are given in Table 5. Details of the X-ray data of **1** will be discussed at the end of this section. A negative CD Cotton effect near 295 nm was observed for **1**, which confirmed the absolute stereochemistry as that shown in structural formula **5**.

From a more polar VLC fraction (hexane−EtOAc, 9:1), a colorless crystalline compound (**3a**, mp 101−103 °C) was obtained that differed from **1** only in the presence of a β-hydroxyl group at C-3 instead of the ketone group in **1**, as indicated by the lack of a carbonyl absorption and the presence of a hydroxyl IR band at 3387 cm^{-1}.

Table 2. ^{1}H-NMR Spectral Data of Compounds **1**, **3a**,**b**, **4**, and **5** (250 MHz, $CDCl_3$)[a]

proton	1	3a	3b	4	5
H-2a	2.26 m			2.39 ddd (3.8, 6.8, 15)	2.31m
H-2b	2.32 m			2.59 ddd (7.0, 11, 15)	2.72ddd (6.7, 14, 14)
H-3		3.28 dd (10.1, 4.7)	4.56 dd (10.1, 5.2)		
H-18	0.99 s	0.80 s	0.84 s	0.74 s	0.91 s
H-19a	0.57 d (4.4)	0.33 d (4.3)	0.36 d (4.3)	1.06 s	0.59 d (4.2)
H-19b	0.78 d (3.6)	0.55 d (4.1)	0.57 d (4.1)		0.79 d (4.2)
H-21	0.89 d (5.5)	0.88 d (6.2)	0.87 d (6.8)	0.98 d (6.4)	0.89 d (6.2)
H-23				4.48 ddd (2.7, 9.1,9.1)	
H-24	2.69 dd (6.2)	2.69 dd (6.1)	2.69 dd (5.9)	5.19 dd (2.2, 9.1)	9.78 dd (1.8,1.8)
H-26	1.26 s	1.26 s	1.27 s	1.69 s	
H-27	1.30 s	1.30 s	1.31 s	1.71 s	
H-28	1.04 s	0.96 s	0.88 s	1.09 s	1.05 s
H-29	1.09 s	0.96 s	0.89 s	1.12 s	1.11 s
H-30	0.90 s	0.89 s	0.96 s	0.88 s	1.00 s
H-2′			2.05 s		

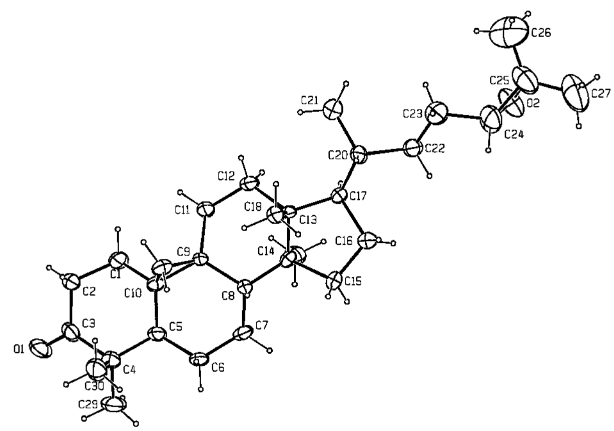
[a] Expressed as δ values in ppm, with *J* values in Hz in parentheses.

Table 3. ^{13}C-NMR Spectral Data of Compounds **1**, **3a**,**b**, **4**, and **5** (62.5 MHz, $CDCl_3$)[a]

carbon	1	3a	3b	4	5
C-1	33.4 t	32.0 t	31.6 t	36.1 t	33.6 t
C-2	37.4 t	30.4 t	26.5 t	34.6 t	37.7 t
C-3	216.5 s	78.8 d	80.7 d	217.7 s	216.7 s
C-4	50.2 s	40.5 s	39.5 s	47.4 s	50.5 s
C-5	48.4 d	48.0 d	47.8 d	50.9 d	48.6 d
C-6	21.5 t	21.1 t	20.9 t	19.4 t	21.7 t
C-7	28.1 t	28.2 t	28.2 t	28.3 t	28.3 t
C-8	47.9 d	47.1 d	47.2 d	133.2 s	48.1 d
C-9	21.1 s	20.0 s	20.1 s	133.8 s	21.3 s
C-10	26.0 s	26.0 s	26.0 s	36.9 s	26.2 s
C-11	25.8 t	26.1 t	25.8 t	21.1 t	26.1 t
C-12	35.5 t	35.5 t	35.5 t	26.3 t	35.7 t
C-13	45.3 s	45.3 s	45.3 s	44.6 s	45.6 s
C-14	48.7 s	48.8 s	48.9 s	50.0 s	49.0 s
C-15	32.8 t	32.9 t	32.9 t	31.0 t	33.0 t
C-16	26.7 t	26.4 t	26.8 s	30.9 t	26.9 t
C-17	52.2 d	52.1 d	52.2 d	51.3 d	52.4 d
C-18	18.1 q	18.0 q	18.0 q	18.1 q	18.2 q
C-19	29.5 t	29.9 t	29.8 t	18.7 q	29.7 t
C-20	35.8 d	35.8 d	35.9 d	33.1 d	35.9 d
C-21	18.3 q	18.3 q	18.3 q	18.7 q	18.3 q
C-22	32.6 t	32.9 t	32.6 t	44.4 t	41.4 t
C-23	25.6 t	25.6 t	25.7 t	66.0 d	28.5 t
C-24	64.7 d	64.8 d	64.8 d	129.1 d	203.3 d
C-25	58.3 s	58.4 s	58.4 s	135.3 s	
C-26	18.7 q	18.7 q	18.8 q	24.2 q	
C-27	24.9 q	24.9 q	24.9 q	26.2 q	
C-28	22.2 q	25.4 q	25.4 q	25.7 q	22.4 q
C-29	20.7 q	14.0 q	15.1 q	15.9 q	21.0 q
C-30	19.2 q	19.3 q	19.3 q	21.3 q	19.5 q
C-1′			170.9 s		
C-2′			21.3 q		

[a] Peak multiplicities were determined by heteronuclear multipulse programs (DEPT); s = singlet, d = doublet, t = triplet, q = quartet.

The empirical formula, $C_{30}H_{50}O_2$, was initially derived from ^{1}H- and ^{13}C-NMR spectral data including DEPT experiments as well as mass spectral values. The mass spectrum gave a parent peak at *m/z* 442, which indicated a molecular weight two mass units higher than **1** and supported the presence of a hydroxyl at C-3 in **3a** instead of the C-3 ketone moiety in **1**. The ^{1}H-



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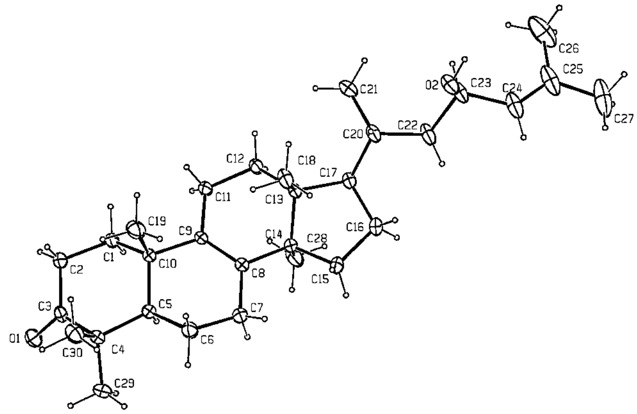

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Figure 1. Molecular structures of compounds **1** and **4**.

Table 4. Crystallographic Data of Triterpenes **1** and **4**

	compd **1**	compd **4**
formula	$C_{30}H_{48}O_2$	$C_{30}H_{48}O_2$
mol wt	440.7	440.7
space grp	monoclinic $P2_1$	monoclinic $P2_1$
a (Å)	7.527(1)	11.926(1)
b (Å)	9.127(1)	7.479(1)
c (Å)	19.664(3)	16.203(2)
β (°)	97.60(1)	112.25(1)
V (Å^3)	1339.0(6)	1337.6(6)
D_c (g cm^3)	1.093	1.094
Z	2	2
μ Cu Kα (cm^{-1})	4.5	4.7
T (°C)	23	22
cryst dimens (mm)	0.25 × 0.22 × 0.05	0.60 × 0.40 × 0.12
cryst	colorless plate	colorless lath
θ range (deg)	2−75	2−75 (hemisphere)
unique data	2935	5440
obsd data	1610	5202
criterion for obsd	$I > 1\sigma(I)$	$I > 3\sigma(I)$
refined variables	289	293
intensity decay (%)	14.0	4.3
min rel tranmission (%)	80.9	93.7
R	0.100	0.055
R_w	0.077	0.075
max resid density (e Å^{-3})	0.31	0.64
min resid density (e Å^{-3})	−0.10	−0.13

NMR spectrum of **3a** (Table 2) gave a signal at δ 3.28 (dd, J = 10.1, 4.7 Hz), which was in agreement with a β-oriented hydroxyl group at C-3.[5] Nearly identical proton signals due to the side chain of **3a**, in particular, the triplet due to H-24 at δ 2.69 (J = 6.1 Hz), indicated that **1** and **3a** must have the same side chain. As in the case of compound **1**, **3a** exhibited a ^{1}H-NMR spectrum with seven methyl absorptions between δ 0.80 and 1.30, and cyclopropane doublets appeared at δ 0.33 and 0.55 (J = 4.2 Hz). ^{13}C-NMR data obtained at 62.5

Table 5. Coordinates and Equivalent Isotropic Thermal Parameters for (24*R*)-24,25-Epoxycycloartan-3-one (**1**)[a]

atom	*x*	*y*	*z*	B_{eq} (Å^2)
O-1	0.5791(6)	0	1.0479(2)	10.4(2)
O-2	0.0685(9)	−0.105(1)	0.2595(3)	12.5(3)
C-1	0.3169(8)	0.026(1)	0.8864(3)	6.7(3)
C-2	0.3696(9)	0.089(1)	0.9576(3)	6.8(2)
C-3	0.5535(9)	0.040(1)	0.9902(3)	6.6(2)
C-4	0.7034(8)	0.053(1)	0.9443(3)	5.8(2)
C-5	0.6380(8)	0.005(1)	0.8711(3)	6.4(2)
C-6	0.7789(8)	0.019(1)	0.8223(3)	7.4(3)
C-7	0.7061(9)	−0.048(1)	0.7531(3)	7.4(3)
C-8	0.5513(8)	0.043(1)	0.7178(3)	5.3(2)
C-9	0.4072(7)	0.077(1)	0.7630(3)	4.7(2)
C-10	0.4564(8)	0.0646(9)	0.8411(3)	4.3(2)
C-11	0.2101(8)	0.040(1)	0.7336(3)	5.6(2)
C-12	0.1564(8)	0.051(1)	0.6570(3)	5.5(2)
C-13	0.3146(7)	0.0814(9)	0.6143(3)	3.6(2)
C-14	0.4720(8)	−0.0134(9)	0.6471(3)	4.3(2)
C-15	0.6052(9)	0.004(1)	0.5925(3)	6.8(2)
C-16	0.4874(9)	0.020(1)	0.5241(3)	6.9(3)
C-17	0.2878(7)	0.0259(9)	0.5392(3)	4.2(2)
C-18	0.3560(8)	0.244(1)	0.6195(3)	4.9(2)
C-19	0.4355(8)	0.209(1)	0.8070(3)	5.7(2)
C-20	0.1757(9)	0.112(1)	0.4842(3)	5.5(2)
C-21	−0.023(1)	0.114(1)	0.4964(4)	7.0(3)
C-22	0.1805(9)	0.028(2)	0.4118(3)	11.3(4)
C-23	0.091(1)	0.091(2)	0.3577(4)	12.4(5)
C-24	0.147(1)	0.018(2)	0.2911(4)	12.3(4)
C-25	0.038(1)	0.043(1)	0.2229(4)	11.4(4)
C-26	−0.146(2)	0.088(2)	0.2174(7)	17.0(5)
C-27	0.140(2)	0.048(2)	0.1638(5)	16.7(5)
C-28	0.426(1)	−0.173(1)	0.6496(4)	6.8(2)
C-29	0.857(1)	−0.042(1)	0.9754(4)	9.0(3)
C-30	0.760(1)	0.212(1)	0.9493(4)	8.1(3)

[a] Figures in parentheses are ESD.

MHz (Table 3) and DEPT experiments confirmed the presence of seven methyls, and instead of the C-3 carbonyl absorption at δ 216.5 in **1**, in **3a** a signal typical of a hydroxyl-bearing carbon absorption appeared at δ 78.8 (C-3). Further ^{1}H- and ^{13}C-NMR signals of **3a** were assigned by inspection and spectral comparison with **1** and a previously isolated mixture of epimers[6] that differed only in the configuration of the epoxide ring at C-24.

Acetylation of **3a** gave the monoacetate **3b**, $C_{32}H_{52}O_3$, which was indicated by a three-proton methyl singlet at δ 2.05 in the ^{1}H-NMR spectrum. In addition, H-3 was shifted from δ 3.28 in **3a** to 4.56 in **3b**, supporting the presence of an acetate group at C-3 in **3b**. The ^{13}C-NMR spectrum (Table 2) of **3b** differed from **3a** only in the presence of two additional signals, a singlet at δ 170.9 and a quartet at δ 21.3, which correspond to the acetate carbonyl and methyl, respectively. All other carbon signals were very similar to those of **3a**, and peak assignments of **3b** were made by correlation with **3a** and related compounds reported in the literature.[6−9]

Chemical correlation of alcohol **3a** with ketone **1** was first attempted by oxidation of **3a** with pyridinium chlorochromate (PCC).[10] However, this reagent not only oxidized the C-3 hydroxyl group of **3a** but also affected the C-24−C-25 epoxide function leading to the fragmentation product **5**, $C_{27}H_{42}O_2$, the structure of which was supported by mass spectral, ^{13}C-NMR, and DEPT data. The mass spectrum of **5** gave a parent peak at m/z 398, and as in **1** loss of the side chain gave a peak at m/z 313. The compound showed IR bands at 1704 and 1726 (sh) cm^{-1} corresponding to two carbonyl absorptions. The ^{1}H-NMR spectrum of **5** indicated the presence of only five methyl groups, with the methyl absorptions corresponding to H-26 and H-27 in **1** being absent in **5** (Table 2). A triplet at δ 9.78 (J = 1.8 Hz) was assigned to an aldehyde proton (H-24), and the two diagnostic cyclopropane doublets appeared at δ 0.59 and 0.79 (J = 4.2 Hz). When compared with data for compound **1**, the ^{13}C-NMR spectrum of **5** suggested the loss of three carbons (C-25, C-26, and C-27). Instead, an aldehyde signal appeared at δ 203.3, which was assigned to C-24. Also, carbon signals of the adjacent positions C-22 (δ 41.4) and C-23 (δ 28.5) were shifted downfield in **5** relative to **1** due to the deshielding effect of the aldehyde carbonyl (C-24). All other ^{13}C-NMR spectral signals of **5** were nearly identical to those of **1**, with assignments being based on comparison with the analog absorptions of **1** and literature values reported for structurally related compounds.[4,6] Selective oxidation of **3a** with $RuCl_3$ and $NaIO_4$[11] provided **1**, which confirmed that the stereogenic center C-24 in **3a** also has the R configuration. Therefore, **3a** differs from **1** only by the presence of a 3β-hydroxyl moiety instead of a keto group.[4,5]

Compound **4**, $C_{30}H_{48}O_2$, showed IR absorptions at 1630 (ketone) and 3435 (hydroxyl) cm^{-1}. When compared with **1**, its ^{1}H-NMR spectrum (Table 2) lacked the diagnostic triplet at δ 2.69 corresponding to H-24 of the epoxide moiety. Instead, two additional signals were present in **4**: a broadened doublet at δ 4.48 (H-23) and a doublet of doublets at δ 5.19 (H-24), which were allylically coupled to two broadened methyl singlets absorbing at δ 1.69 and 1.71. This indicated the absence of an epoxide and strongly suggested the presence of a double bond in the side chain of **4**. The diagnostic cyclopropane methylene proton doublets at δ 0.57 and 0.78 in **1** were also absent in **4**. Instead, compound **4** contained eight methyl signals, indicating one additional methyl group, when compared with **1**. The ^{13}C-NMR spectrum of **4** (Table 2) and DEPT experiments supported the absence of the cyclopropane ring at C-19. Furthermore, the presence of a C-3 keto group (δ 217.7), eight methyl quartets, and four olefinic carbons was established. Spectral comparison with literature values suggested a lanostadiene-type skeleton.[9] The mass spectrum gave a molecular ion peak at m/z 440 and the typical [M − side chain]$^+$ peak at m/z 313. NMR spectral assignments of **4** were made by spectral comparison with data described in the literature for structurally closely related compounds.[6,8] The molecular structure of **4** (Figure 1) was determined by single-crystal X-ray diffraction techniques. Table 4 summarizes the crystallographic data, and its coordinates are listed in Table 6. A negative Cotton effect in the CD spectrum was observed near 295 nm, which confirmed the absolute stereochemistry as shown in structural formula **4**.[5]

Details of data collection and refinements made in the X-ray diffraction analysis of **1** and **4** are given in Table 4. Figure 1 illustrates the α-oriented epoxide at C-24−C-25 and a β-cyclopropane ring fused at C-9−C-10 in **1** and the OH group at C-23 and C-8═C-9 double bond in **4**. The C8═C9 distance in **4** is 1.352(3) Å. The quality of the crystal of **4** was much higher than that of **1**, and thus the precision of the determination was also much higher. This is apparently a result of hydrogen bonding in **4**. The OH group O-2 is engaged in a linear, intermolecular hydrogen bond with carbonyl oxygen O-1 (at $1 + x, y, 1 + z$) as acceptor. The O···O distance is 2.886(2) Å, and the angle about the H atom is 173(3).

Table 6. Coordinates and Equivalent Isotropic Thermal Parameters for (23*R*)-3-Oxolanosta-8,24-dien-23-ol (**4**)[a]

atom	x	y	z	B_{eq} (Å^2)
O-1	0.1260(2)	0	0.4697(1)	5.32(4)
O-2	0.9858(1)	−0.1580(3)	1.29915(9)	4.74(4)
C-1	0.3570(2)	0.1313(3)	0.6674(1)	3.91(4)
C-2	0.2277(2)	0.1657(3)	0.6007(1)	4.38(5)
C-3	0.1714(2)	−0.0016(3)	0.5509(1)	3.55(4)
C-4	0.1745(2)	−0.1674(3)	0.6049(1)	3.38(4)
C-5	0.3047(2)	−0.1875(3)	0.6787(1)	3.09(3)
C-6	0.3180(2)	−0.3464(3)	0.7402(2)	4.41(5)
C-7	0.4512(2)	−0.3891(3)	0.7886(2)	4.48(5)
C-8	0.5303(2)	−0.2287(3)	0.8222(1)	3.13(4)
C-9	0.4920(2)	−0.0602(3)	0.7960(1)	2.96(3)
C-10	0.3615(2)	−0.0184(3)	0.7335(1)	3.10(3)
C-11	0.5769(2)	0.0979(3)	0.8297(1)	3.91(4)
C-12	0.7006(2)	0.0658(3)	0.9056(1)	4.15(5)
C-13	0.7062(1)	−0.1103(3)	0.9542(1)	2.95(3)
C-14	0.6613(2)	−0.2593(3)	0.8818(1)	3.14(4)
C-15	0.6918(2)	−0.4288(3)	0.9391(2)	5.08(6)
C-16	0.8132(2)	−0.3864(3)	1.0152(1)	4.17(5)
C-17	0.8335(2)	−0.1816(3)	1.0160(1)	3.23(4)
C-18	0.6243(2)	−0.0952(4)	1.0071(1)	4.29(4)
C-19	0.2983(2)	0.0462(4)	0.7962(2)	5.37(6)
C-20	0.8933(2)	−0.1043(3)	1.1105(1)	3.68(4)
C-21	0.9174(2)	0.0952(4)	1.1108(2)	5.05(5)
C-22	1.0110(2)	−0.2084(4)	1.1615(1)	4.90(5)
C-23	1.0750(2)	−0.1614(4)	1.2601(1)	4.88(5)
C-24	1.1665(2)	−0.3097(5)	1.3066(2)	6.22(7)
C-25	1.2792(2)	−0.2982(7)	1.3619(2)	7.68(9)
C-26	1.3437(3)	−0.1212(9)	1.3866(2)	10.9(1)
C-27	1.3502(3)	−0.4605(8)	1.4067(2)	11.1(1)
C-28	0.7346(2)	−0.2646(4)	0.8211(1)	5.28(5)
C-29	0.1461(2)	−0.3306(3)	0.5435(2)	4.72(5)
C-30	0.0731(2)	−0.1495(4)	0.6413(2)	4.88(5)

[a] Figures in parentheses are ESD.

Table 7. Minimum Inhibitory Concentrations Against *M. tuberculosis* and IC_{50} Values against Vero cells

compd	MIC (μg/mL)	IC_{50} (μg/mL)
1	8	71.8
3a	8	39.8
3b	>128	
4	64−128	103.6
6	>128	
7	>128	
fusidic acid	4	105.8

A search of the Cambridge Crystallographic Database[13] yielded no previous crystal structure determinations for triterpenes with the cycloartane or lanostadiene skeletons having an epoxide at C24−C25. The compounds most closely related to **1** for which crystal structures have been determined are 3-oxo-24-cycloarten-21-oic acid[14] and argentatin C,[15] which differ from **1** only by having a glycol at C-24−C-25 rather than an epoxide and by having a β-OH group at C-16. The compounds most closely related to **4** for which crystal structures have been determined are euphyl acetate and tirucallyl acetate.[16] Both have acetate substituents at C-3 and lack the OH group at C-23.

In a radiorespirometric bioassay against *M. tuberculosis* ($H_{37}Rv$),[3] both triterpenes **1** and **3a** showed minimum inhibitory concentrations (MICs) of 8 μg/mL, while compounds **4** and **3b** had MIC values of 64−128 μg/mL and >128 μg/mL, respectively (Table 7). Correlations of structural features and the MICs of the four triterpenes suggest that the presence of the C-3 keto and/or β-hydroxy group, the cyclopropane ring, and the epoxide moieties as in **1** and **3a** seem to play a major role in the *in vitro* antituberculosis activity. Both the cyclopropane and epoxide functions are absent in triterpene **4**, resulting in its loss of activity (MIC 64−128 μg/mL). Also, the loss of activity by introduction of a C-3 acetoxy group (MIC of **3b** >128 μg/mL) strongly suggests that either a free hydroxyl or a keto group at C-3 in **1** or **3a** is required for significant activity. Argentatine A (**6**) and B (**7**) were also tested against *M. tuberculosis* due to their structural and biosynthetic similarities to the bioactive natural triterpenes.[4] The lack of significant activity of compounds **6** and **7** with MIC's >128 μg/mL (Table 7) suggests that the epoxide ring present in the side chain of the active triterpenes **1** and **3a** appears to be essential for the *in vitro* antituberculosis activity. These preliminary structure–activity data require further verification by testing structurally related triterpenes to learn about the essential active regions necessary for significant antituberculosis activities. The clinically active triterpene fusidic acid was also tested against *M. tuberculosis* for comparison. In our radiorespirometric bioassay, its MIC of 4 μg/mL was lower than the previously reported value of 32–64 μg/mL.[17] Cytotoxicity results (Table 7) for a mammalian cell line indicate that the active triterpenes **1** and **3a** have IC_{50} values of 71.8 and 39.8 μg/mL, respectively, while the inactive triterpene **4** has an IC_{50} of 103.6 μg/mL, suggesting some degree of selective toxicity for *M. tuberculosis*.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker AM 250 MHz spectrometer. Mass spectra were obtained on a Hewlett-Packard 5971A GC-MS or a TSQ70 FAB mass spectrometer. IR spectra were run on a Perkin-Elmer 1760X spectrometer as a film on KBr plates. Vacuum–liquid chromatographic (VLC) separations were carried out on silica gel (MN Kieselgel).

Plant Material. *B. frutescens* was collected in May 1994 in a brackish environment about 1 mile inland from the Gulf of Mexico at Grand Isle, LA (N. H. Fischer No. 501; voucher deposited at LSU Herbarium).

Extraction and Isolation. Air-dried flowers (910 g) of *B. frutescens* were extracted at room temperature with 1700 mL of hexane. Evaporation of the solvent *in vacuo* provided 10.1 g of crude extract. The plant residue was then extracted with CH_2Cl_2 (2 × 1800 mL for 24 h) to yield, after removal of solvent, 19.8 g of crude CH_2Cl_2 extract. Biological screening of the extracts led to the investigation of the CH_2Cl_2 extract, 9.8 g of which was adsorbed on 8 g of Si gel and placed onto a VLC column (4 cm in diameter and 30 cm long) packed with 70 g of Si gel.[18] The extract was separated into eight fractions using a gradient of hexane–EtOAc–MeOH of increasing polarity. Table 1 lists the amounts and proportions of solvents used in the fractionations as well as the percent inhibition of each fraction against *M. tuberculosis*. On the basis of these results, fractions 2–4 were used for further isolation of the active constituents.

Fraction 3 (1.8 g) was adsorbed on 3 g of silica gel and placed onto a VLC column (3 cm in diameter and 25 cm long) packed with 40 g of Si gel and chromatographed using 11 × 100 mL hexane–EtOAc mixtures of increasing polarity. Altogether, 53 fractions (20 mL each) were collected. Fractions 11–14 (hexane–EtOAc, 95:5) were evaporated to provide 145 mg of crystalline **1**. Fractions 21–23 (hexane–EtOAc, 92:8) gave 30 mg of crystalline **4**. Fractions 28 and 29 (hexane–EtOAc, 9:1) slowly crystallized from a mixture of hexane and EtOAc (85:5) to yield 41 mg of **3a**.

(24*R*)-24,25-Epoxycycloartan-3-one (1): colorless crystals; $C_{30}H_{48}O_2$; mol wt 440.717; mp 119–122 °C; IR ν_{max} (KBr) 1708 (C=O) cm^{-1}; CD nm (ϵ) 212 (−1.8), 225 (−18.2), 236 (−0.5), 298 (−32.6), 401 (−0.1) (*c* 0.0019; MeOH); ^{1}H-NMR spectral data see Table 1; ^{13}C-NMR spectral data see Table 2; EIMS (70 eV) *m/z* $[M]^+$ 440 (7), $[M - Me]^+$ 425 (4), $[M - H_2O]^+$ 422 (1), $[M - \text{side chain}]^+$ 313 (40), 302 (16), 175 (35), 163 (24), $[\text{side chain}]^+$ 127 (13), 121 (50), $[127 - H_2O]^+$ 109 (53), 107 (56), $[127 - Me - OH]^+$ 95 (100), 81 (52), 69 (60), 55 (76), 43 (74).

(3β,24*R*)-24,25-Epoxycycloartan-3-ol (3a): colorless crystals; $C_{30}H_{50}O_2$; mol wt 442.732; mp 101–103 °C; IR ν_{max} (KBr) 3387 (OH) cm^{-1}; CD nm (ϵ) 201 (−0.06), 222 (+19.1), 244 (−0.01), 307 (−2.9), 396 (−0.03) (*c* 0.0005; MeOH); ^{1}H-NMR spectral data see Table 1; ^{13}C-NMR spectral data see Table 2; EIMS (70 eV) *m/z* $[M - H_2O]^+$ 424 (8), $[M - Me - OH]^+$ 410 (10), $[424 - Me]^+$ 409 (18), $[M - \text{side chain}]^+$ 315 (9), 311 (20), $[315 - H_2O]^+$ 297 (47), 260 (10), 258 (17), 241 (14), 227 (14), 203 (48), $[\text{side chain} - H_2O]^+$ 109 (64), 107 (100), 91 (79), 81 (91), 79 (78), 69 (69), 55 (98.6), 43 (99); FABMS *m/z* $[M]^+$ 442.6, $[M - H]^+$ 441.7, $[M - OH]^+$ 425.5, $[M - Me - H_2O]^+$ 409.7.

(23*R*)-3-Oxolanosta-8,24-dien-23-ol (4): colorless crystals; $C_{30}H_{48}O_2$; mol wt 440.717; mp 73–75 °C; IR ν_{max} (KBr) 1630 (C=O), 3435 (OH) cm^{-1}; CD nm (ϵ) 215 (0.0), 227 (−4.4), 275 (0.0), 311 (−3.7), 369 (−0.1), (*c* 0.0005; MeOH); ^{1}H-NMR spectral data see Table 1; ^{13}C-NMR spectral data see Table 2; EIMS (70 eV) *m/z* $[M - OH]^+$ 423 (3), $[M - H_2O]^+$ 422 (8), $[423 - M]^+$ 408 (4), $[422 - M]^+$ 407 (14), $[M - \text{side chain}]^+$ 313 (16), 271 (13), 257 (13), $[\text{side chain}]^+$ 127 (1), $[127 - OH]^+$ 110 (12), $[127 - H_2O]^+$ 109 (100), 81 (50), 69 (29), 67 (35), 55 (41), 43 (31), 41 (31).

Acetylation of 3a. Compound **3a** (20 mg) was dissolved in 1 mL of pyridine. After addition of 1 mL of Ac_2O the mixture was allowed to react at room temperature for 12 h. After removal of the reagents *in vacuo*, the residue was separated by VLC (2 cm inner diameter column, 8 g of silica gel) using a gradient elution with hexane and hexane–EtOAc mixtures of increasing polarity. This yielded 11 mg of pure crystalline **3b**: colorless crystals; $C_{32}H_{52}O_3$; mol wt 484.37; mp 161–163 °C; IR ν_{max} (KBr) 1726 (C=O) cm^{-1}; CD nm (ϵ) 207 (0.4), 226 (−6.56), 257 (0.0), (*c* 0.0012; MeOH); ^{1}H-NMR spectral data see Table 1; ^{13}C-NMR spectral data see Table 2; EIMS (70 eV) *m/z* $[M]^+$ 484 (1), $[M - AcOH]^+$ 424 (2), $[424 - Me]^+$ 409 (2), 302 (4), $[424 - \text{side chain}]^+$ 297 (3), 175 (20), 161 (13), 135 (25), $[\text{side chain}]^+$ 127 (10), 107 (35), 95 (39), 69 (42), 59 (15), $[Ac]^+$ 43 (100).

Pyridinium Chlorochromate (PCC) Oxidation of 3a to 5. Compound **3a** (48 mg), dissolved in 3 mL of CH_2Cl_2, was added to a solution of PCC (100 mg) in CH_2Cl_2 (25 mL), and the mixture was allowed to react for 1 h. After addition of 10 mL of Et_2O, the reaction mixture was adsorbed onto silica gel and separated by VLC (2.3 cm inner diameter column, 6 g of silica gel) using a gradient elution of hexane or hexane–EtOAc of increasing polarity to yield 9 mg of pure, crystalline **5**: gum; $C_{27}H_{52}O_2$; mol wt 389.63; IR ν_{max} (KBr) 1704 (C=O), 1726 (C=O sh) cm^{-1}; ^{1}H-NMR spectral data see Table 1; ^{13}C-NMR spectral data see Table 2; EIMS (70 eV) *m/z* $[M]^+$ 398 (11), $[M - CH_3]^+$ 383 (7), $[M - H_2O]^+$ 380 (7), $[380 - Me]^+$ 365 (3), $[M - \text{side chain}]^+$ 313 (27), 175

(39), 161 (49), 147 (36), 133 (49), 121 (62), 107 (71), 95 (98), [side chain]$^+$ 85 (37), 81 (73), 67 (74), 55 (100).

$RuCl_3$/$NaIO_4$ Oxidation of 3a to 1. To a solution of 47.4 mg of **3a** in 2 mL of CH_3CN, 2 mL of CCl_4, and 3 mL of H_2O was added 85.6 mg (4 equiv) of sodium metaperiodate.[11] To this biphasic solution was added 1.1 mg (4.1 mol %) of ruthenium trichloride hydrate and the mixture stirred magnetically for 8 h while the reaction was monitored by TLC. At completion of the reaction, 10 mL of CH_2Cl_2 was added to this solution, and the phases were separated. The aqueous layer was extracted three times with a total of 30 mL of CH_2Cl_2. The organic extracts were combined, dried ($MgSO_4$), and concentrated. The residue was diluted with 20 mL of ether and filtered through Celite.[11] The remaining material was adsorbed onto silica gel and separated by VLC to yield 11.3 mg of pure **1**.

X-ray Crystallographic Analysis.[19] Intensity data for **1** and **4** were collected on an Enraf-Nonius CAD4 diffractometer equipped with Cu Kα radiation (λ = 1.541 84 Å), and a graphite monochromator, by $\omega - 2\theta$ scans of variable rate. Data reduction included corrections for background, Lorentz, polarization, decay, and absorption effects. Absorption corrections were based on ψ scans, and linear corrections were made for decay. The structures were solved by direct methods and refined by full-matrix least-squares techniques, treating non-hydrogen atoms anisotropically, using the Enraf-Nonius MolEN programs.[12] Hydrogen atoms were placed in calculated positions, except for that of the OH group of **4**, which was refined isotropically. Details of data collections and refinements are given in Table 4.

Radiorespirometric Bioassays. All compounds were solubilized at 10.24 mg/mL in DMSO, filter sterilized, and stored at −80 °C until used. Subsequent dilution was done in DMSO. Fifty microliters of each solution were added to 4 mL of BACTEC 12B broth (Becton Dickinson, Towson, MD) to achieve the desired final concentrations.

MIC's were performed in the BACTEC 460 essentially as described by Heifets.[3] *M. tuberculosis* $H_{37}Rv$ was cultured in 4 mL of BACTEC 12B broth until a daily growth index (GI) of 400−999 was reached. One tenth mL of this was used to inoculate 4 mL of fresh BACTEC 12B medium containing test compounds. Additional controls diluted 1:100 were also included. Cultures were incubated at 37 °C, and the GI was determined daily starting on the third day of incubation. Percent inhibition of fractions was calculated as (1 − GI test sample/GI undiluted control) × 100 on the day that the undiluted controls reached peak values of 999. The minimum inhibitory concentration (MIC) of pure compounds was defined as the lowest concentration of drug that effected a daily GI increase and final GI lower than the 1:100 diluted control vial readings when the 1:100 GI was >30. This corresponds to the concentration that inhibited the growth of 99% of the organisms. Experiments were usually completed within 10 days.

Cytotoxicity Assay. Test compounds were dissolved at 20−40 mg/mL in ethanol. Geometric three-fold dilutions were performed in growth medium M199 [Gibco, Grand Island, NY] + 5% fetal bovine serum [HyClone, Logan, UT] +25 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid [HEPES, Gibco] + 0.2% $NaHCO_3$ [Gibco] + 2 mM glutamine [Irvine Scientific, Santa Ana, CA] to achieve final concentrations ranging from 4.2 to 400 μg/mL. Final ethanol concentrations did not exceed 1% v/v. Drug dilutions were distributed in duplicate in 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at a volume of 50 μL/well. An equal volume containing 5×10^3 log phase Vero cells (CCL-81; American Type Culture Collection, Rockville, MD) was added to each well, and the cultures were incubated at 37 °C in an atmosphere of 5% CO_2 in air. After 72 h, cell viability was measured using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Absorbance at 490 nm was read in a BioRad Model 3550 microplate reader (Hercules, CA). The IC_{50} is defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells. Maximum cytotoxicity (100%) was determined by lysing the cells with sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, MO).

Acknowledgment. Financial support from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, under Contract No. Y02-AI-30123 is gratefully acknowledged. We thank Anita N. Biswas and Ken E. White for technical assistance.

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(19) Atomic coordinates, thermal parameters, bond distances and angles, and observed and calculated structure parameters have been deposited with the Cambridge Crystallographic Data Centre and can be obtained upon request from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge CB2 IEZ, UK.

NP960551W