Sesquiterpenoids from *Tithonia diversifolia* with Potential Cancer Chemopreventive Activity

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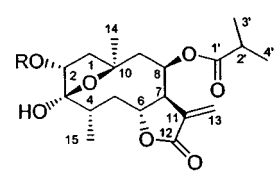
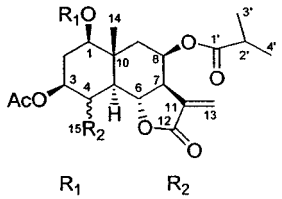
Activity-guided fractionation of an ethyl acetate extract of the aerial parts of *Tithonia diversifolia*, using an antiproliferation bioassay performed with human colon cancer (Col2) cells, led to the isolation of three new sesquiterpenoids, 2α-hydroxytirotundin (**1**), tithofolinolide (**2**), and 3α-acetoxydiversifolol (**3**), along with eight known sesquiterpene lactones, 3*β*-acetoxy-8*β*-isobutyryloxyreynosin (**4**), tagitinin C (**5**), 1*β*,2α-epoxytagitinin C (**6**), 4α,10α-dihydroxy-3-oxo-8*β*-isobutyryloxyguaia-11(13)-en-12,6α-olide (**7**), 3α-acetoxy-4α-hydroxy-11(13)-eudesmen-12-oic acid methyl ester, 17,20-dihydroxygeranylnerol, tagitinin A, and tirotundin. These isolates were evaluated for their potential as cancer chemopreventive agents, by measuring antiproliferative activity in Col2 cells and induction of cellular differentiation in human promyelocytic leukemia (HL-60) cells. Selected compounds were then investigated for their ability to inhibit 7,12-dimethylbenz[*a*]anthracene-induced preneoplastic lesions in a mouse mammary organ culture assay. Among these isolates, **5** and **6** showed significant antiproliferative activity, **2**, **4**, and **7** induced HL-60 cellular differentiation, and **4** significantly inhibited (63.0% at 10 *μ*g/mL) lesion formation in the mouse mammary organ culture assay. The chemical structures of **1**−**3** were elucidated by spectroscopic analysis. The absolute configurations of **1** and **2** were determined by Mosher ester methodology.

Tithonia diversifolia (Hemsl.) A. Gray (Asteraceae) is a 2−5 m tall perennial shrub, which is native to Mexico and also grows in parts of Africa, Australia, Asia, and other countries of North America, where it may become locally abundant.[1,2] Extracts of *T. diversifolia* have been used traditionally for the treatment of diarrhea, fever, hematomas, hepatitis, hepatomas, malaria, and wounds.[3−5] Previous phytochemical investigations on *T. diversifolia* resulted in the isolation of cadinane, chromene, eudesmane, flavone, germacrane, and rearranged eudesmane derivatives.[6−13]

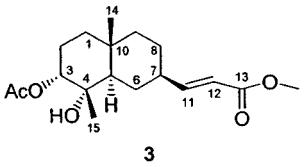
As part of an ongoing collaborative search for novel plant-derived cancer chemopreventive agents,[14,15] a methanolic extract of the aerial parts of *T. diversifolia* was found to exhibit several significant biological activities in preliminary screening, inclusive of antiproliferative activity performed in a sulforhodamine B assay with human colon cancer (Col2) cells,[16] cellular differentiation-inducing activity with human promyelocytic leukemia (HL-60) cells,[17] and inhibitory activity in a 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mouse mammary organ culture assay system.[18] Bioassay-guided fractionation of an active ethyl acetate-soluble partition using the above-mentioned antiproliferation assay led to the isolation of three new sesquiterpenoids (**1**−**3**) and eight known terpenoids. The structure elucidation of **1**−**3** and the biological evaluation of these isolates are described herein.



	R
1	H
1s	(*S*)-MTPA
1r	(*R*)-MTPA



	R_1	R_2
2	H	αOH, βCH_3
2s	(*S*)-MTPA	αOH, βCH_3
2r	(*R*)-MTPA	αOH, βCH_3
4	H	=CH_2



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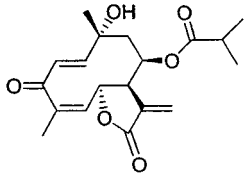

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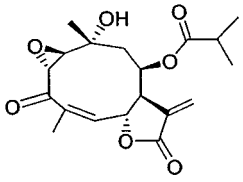

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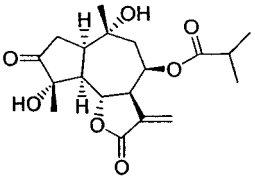

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Results and Discussion

Eight compounds of previously known structure were isolated from the EtOAc extract of the aerial parts of *T.*

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Table 1. NMR Spectral Data for **1**, **1s**, and **1r**[a]

	1					**1s**	**1r**	
position	δ_C	δ_H	COSY	ROESY	HMBC	δ_H	δ_H	$\Delta\delta_{S-R}$
1 α	49.3 t[b]	1.90 dd (2.7, 14.3)	1*β*, 2*β*	1*β*, 2*β*, 14	2, 3, 9	2.01	1.88	+0.13
β		2.30 dd (6.9, 14.2)	1α, 2*β*	1α, 2*β*	3, 9, 10, 14	2.52	2.43	+0.09
2 *β*	74.6 d	4.08 dd (2.7, 6.9)		1*β*, 4*β*, 15	3, 10	5.50	5.46	*R*[d]
3	109.1 s							
4 *β*	41.2 d	1.99 dq (7.0, 11.0)	15	1*β*, 2*β*, 6*β*, 15	6, 15	2.12	2.13	−0.01
5 α	39.9 t	2.18 ddd (9.7, 11.0, 14.2)	6*β*	5*β*, 7α	6	2.13	2.13	
β		1.79 dd (1.0, 14.2)	6*β*	4*β*, 5α, 6*β*, 15	3, 4, 6, 7, 15	1.80	1.79	+0.01
6 *β*	82.6 d	4.67 ddd (1.0, 6.2, 9.7)	5α, 7α	4*β*, 7α, 9	8	4.70	4.70	
7 α	49.4 d	4.19 m (3.3, 6.2)	6*β*, 8α	5α, 6*β*, 8α		4.14	4.14	
8 α	71.3 d	5.55 ddd (3.3, 7.0)	9	7α, 14	6	5.55	5.55	
9	44.4 t	1.85[c]	8α		1, 8, 10, 14	1.93	1.89	+0.04
10	77.7 s							
11	138.8 s							
12	171.6 s							
13 a	122.5 t	5.62 d (2.9)	13b	13b	7, 11, 12	6.18	6.17	+0.01
b		6.18 d (2.9)	13a	13a	7, 11	5.61	5.60	+0.01
14	28.2 q	1.44 s		8α, 1α	1, 9, 10	1.45	1.27	+0.18
15	19.6 q	1.06 d (7.0)				1.13	1.13	
1′	177.5 s							
2′	35.3 d	2.43 sept (7.0)		3′/4′	1′, 3′/4′	2.44	2.43	+0.01
3′/4′	19.3 q	1.05 d (7.0)		2′	1′, 2′	1.06	1.05	+0.01
4′/3′	19.1 q	1.03 d (7.0)		2′	1′, 2′	1.03	1.03	

[a] Spectra obtained at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR in CD_3OD. Figures in parentheses are coupling constants in Hz. [b] Carbon multiplicities were determined according to DEPT and HMQC spectra. [c] Multiplicity patterns were unclear due to signal overlapping. [d] Absolute configuration.

diversifolia, as described in the Experimental Section, and were identified, in turn, as 3*β*-acetoxy-8*β*-isobutyryloxy-reynosin (**4**),[11] tagitinin C (**5**),[13] 1*β*,2α-epoxytagitinin C (**6**),[19] 4α,10α-dihydroxy-3-oxo-8*β*-isobutyryloxyguaia-11(13)-en-12,6α-olide (**7**),[19] 3α-acetoxy-4α-hydroxy-11(13)-eudesmen-12-oic acid methyl ester,[20] 17,20-dihydroxygeranylnerol,[21] tagitinin A,[13] and tirotundin,[22] by comparison of their physical and spectral data with reported values. Among these known isolates, **7** was isolated from this species for the first time, and **5** was the major compound found in the present investigation, in a yield of greater than 0.16% w/w of the dried plant material.

Compound **1**, $[\alpha]^{25}_D$ −78.0° (*c* 0.11, MeOH), was obtained as a light yellow solid. The molecular formula was deduced as $C_{19}H_{28}O_7$ from the HRFABMS data at *m*/*z* 369.1900 $[M + H]^+$ ($C_{19}H_{29}O_7$, calcd for 369.1913). Analysis of its IR spectrum suggested that it contained one or more hydroxyl groups (3480 and 3452 cm^{-1}), an ester (1734 cm^{-1}), and an α-methylene-*γ*-lactone (1756 and 1658 cm^{-1}).[7] The UV absorption at λ_{max} 211 nm was consistent with the presence of the latter functionality. By comparison of the ^{1}H and ^{13}C NMR spectra (Table 1) of **1** with those of tirotundin, which, as previously indicated, was also obtained in the present investigation, it was apparent that **1** contained an additional hydroxyl group [δ_H 4.08 (dd, J = 2.7, 6.9), δ_C 74.6 (d)]. The hydroxyl group in **1** was placed at C-2, as a result of the observed cross-peaks (H_2-1/C-2,-3,-9,-10,-14, and H-2/C-3,-10) in the HMBC spectrum of **1** (Table 1). The relative stereochemistry of **1** was determined based on a ROESY experiment (Figure 1), which gave diagnostic correlations from H-2*β* to H-1*β*, H-4*β*, and H-15, H-4*β* to H-1*β* and H-6*β*, and H-7α to H-5α and H-8α, leading to the assignments of H-2*β*, H-4*β*, H-6*β*, H-7α, and H-8α, respectively. The absolute configuration of the stereogenic center at C-2 in **1** was determined using Mosher ester methodology.[23] Compound **1** was treated with (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride to obtain the (*S*)- (**1s**) and (*R*)-esters (**1r**) (see Experimental Section). Analysis of the $\Delta\delta_{H(S-R)}$ data (Table 1) showed positive differences in chemical shift for the protons at C-1 and C-14 and a negative effect for the proton at C-4. These

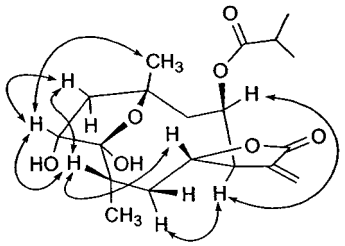

Figure 1. Selected ROESY correlations for **1**.

effects indicated that the absolute configuration at C-2 is *R*. Thus, the structure of **1** was determined as (2*R*)-2,3-dihydroxy-3,10-epoxy-8-isobutyloxygermacra-11(13)-en-6,12-olide (2α-hydroxytirotundin).

Compound **2**, $[\alpha]^{25}_D$ −33.5° (*c* 0.47, MeOH), was obtained as a colorless gel. The molecular formula was deduced as $C_{21}H_{30}O_8$ from the HRFABMS data at *m*/*z* 411.2043 $[M + H]^+$ ($C_{21}H_{31}O_8$, calcd for 411.2019). Analysis of the UV and IR spectra of **2** suggested that it also contained one or more hydroxyl groups, an ester, and an α-methylene-*γ*-lactone. By comparison of the ^{1}H and ^{13}C NMR spectra (Table 2) of **2** with those of 3*β*-acetoxy-8*β*-isobutyryloxyreynosin (**4**),[11] compound **2** exhibited signals for one tertiary methyl group [δ_H 1.44 (s, H_3-15), δ_C 19.2 (C-15)] and one oxygen-substituted carbon [δ_C 73.6 (C-4)], in comparison to the resonances observed for a terminal methylene group in the latter compound. The acetoxyl group in **2** was located at C-3 as a result of the observed HMBC spectral cross-peaks between H-3 and the acetyl carbonyl signal at δ_C 170.6 (Table 2). The relative stereochemistry of **2** was determined to be H-1α, H-3α, H-6*β*, H-7α, H-8α, CH_3-4*β*, and CH_3-10*β*, on the basis of the spatial correlations between H-5α and H-1α, H-3α and H-7α, and H-6*β* and H-14 and H-15 observed in the ROESY spectrum (Figure 2). Moreover, the stereochemistry at H-1α and H-3α was confirmed by the observed coupling constant values ($J_{1\alpha,2\beta}$ = 11.6 Hz, $J_{2\beta,3\alpha}$ = 12.5 Hz). The absolute configuration of the stereogenic center at C-1 in **2** was determined using Mosher ester methodology. Analysis of the $\Delta\delta_{H(S-R)}$ data between the (*S*)-(**2s**) and (*R*)-ester (**2r**) (Table 2) showed a positive difference

Table 2. NMR Spectral Data for **2**, **2s**, and **2r**[a]

	2					**2s**	**2r**	
position	δ_C	δ_H	COSY	ROESY	HMBC	δ_H	δ_H	$\Delta\delta_{S-R}$
1 α	75.8 d[b]	3.64 dd (3.9, 11.6)	2α, 2β	2α, 3α, 5α, 9α	9, 14	5.47	5.47	*R*[c]
2 α	33.1 t	2.06 ddd (3.9, 4.9, 15.0)	1α, 2β, 3α	2β	1, 3, 4	2.53	2.52	+0.01
β		1.71 ddd (12.5, 11.6, 15.0)	1α, 2β, 3α	2α	1, 3, 4, 10	2.18	2.02	+0.16
3 α	75.9 d	4.85 dd (4.9, 12.5)	2α, 2β	1α, 2α, 5α	2, 4, 15, *C*OCH$_3$	5.52	5.47	+0.05
4	73.6 s							
5 α	54.8 d	1.90 d (11.2)	6β	1α, 3α, 7α, 9α	7, 10, 14, 15	2.58	2.62	−0.04
6 β	75.9 d	4.61 dd (11.2, 11.2)	5α, 7α	7α, 14, 15	7, 8, 10, 11	4.91	4.96	−0.05
7 α	53.1 d	2.91 dd (2.7, 11.2)	6β, 8α	5α, 8α, 9α, 13	11	3.28	3.32	−0.04
8 α	65.7 d	5.73 ddd (2.5, 2.7, 2.9)	7α, 9α, 9β	7α, 9α, 9β, 13	6, 10	5.80	5.83	−0.03
9 α	43.4 t	1.57 dd (2.9, 15.0)	8α, 9β	1α, 9β	1, 10, 14	1.92	2.27	−0.35
β		2.29 dd (2.5, 15.0)	8α, 9α	9α, 14	5, 7, 8, 10, 14			
10	42.1 s							
11	133.0 s							
12	168.6 s							
13 a	120.7 t	5.49 d (2.9)	13b	7α, 13b	7, 8	5.47	5.47	-
b		6.21 d (2.9)	13a	13a	7	6.24	6.26	−0.02
14	16.1 q	1.15 s			1, 2, 5, 10	1.26	1.35	−0.19
15	19.2 q	1.44 s		6β	3, 4, 5	1.67	1.66	+0.01
*C*OCH$_3$	170.6 s							
CO*C*H$_3$	21.3 q	2.11 s			*C*OCH$_3$	2.05	2.04	+0.01
1′	176.1 s							
2′	34.3 d	2.52 sept (7.0)	3′/4′	3′/4′	1′, 3′/4′	2.51	2.52	−0.01
3′/4′	19.1 q	1.15 d (7.0)	2′	2′	1′, 2′	1.09	1.08	+0.01
4′/3′	18.9 q	1.14 d (7.0)	2′	2′	1′, 2′	1.08	1.07	+0.01

[a] Spectra obtained at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR (**2** in $CDCl_3$, **2s** and **2r** in C_5D_5N). Figures in parentheses are coupling constants in Hz. [b] Carbon multiplicities were determined according to DEPT and HMQC spectra. [c] Absolute configuration.

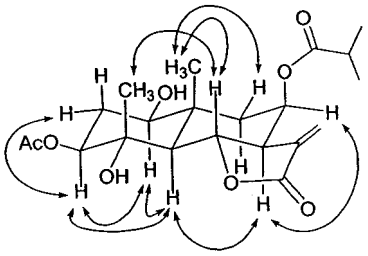

Figure 2. Selected ROESY correlations for **2**.

in chemical shift for the protons at C-2 and C-3 and a negative effect for the protons at C-9 and C-14. These effects indicated that the absolute configuration at C-1 is *R*. Accordingly, the absolute configurations at C-3 and C-4 were deduced as 3*S* and 4*S*, respectively, on the basis of their relative configurations. Thus, the structure of **2** was determined as (1*R*,3*S*,4*S*)-3-acetoxy-1,4-dihydroxy-8-isobutyloxyeudesm-11(13)-en-6,12-olide, to which the trivial name tithofolinolide has been accorded.

Compound **3**, $[\alpha]^{25}_D$ −71.8° (*c* 0.071, MeOH), was obtained as a colorless gel. The molecular formula was deduced as $C_{18}H_{28}O_5$ from the HRFABMS data at *m*/*z* 347.1836 $[M + Na]^+$ ($C_{18}H_{28}O_5Na$, calcd for 347.1834). This molecule contained a hydroxyl group (3512 cm^{-1}), two tertiary methyl groups [δ_H 0.92 and 1.15 (3H each, s)], and a *trans* monosubstituted conjugated ester [1723 and 1658 cm^{-1}; δ_H 6.96 (1H, dd, J = 6.6, 15.8 Hz), 5.82 (1H, dd, J = 1.3, 15.8 Hz), and 3.73 (3H, s)]. The UV spectrum was consistent with the presence of the latter functionality.[8] By comparison of the ^{1}H and ^{13}C NMR data of **3** (Table 3) and the rearranged eudesmane derivative, diversifolol,[8] **3** contained an additional acetoxy group [δ_H 2.12 (3H, s), δ_C 21.4 (q) and 170.7 (s)] compared with the latter compound. The methyl (*E*)-propenoate moiety was placed at C-7 as a result of cross-peaks (H-11/C-8,-13, and H-12/C-7,-13) that appeared in the HMBC spectrum of **3**. The relative stereochemistry of **3** was established as H-1α, H-3β, H-5α, H-7α, CH$_3$-4β, and CH$_3$-10β, on the basis of observed ^{1}H NMR coupling constants, as well as cross-peaks between H-2β and H-1β and H-15, H-14 and H-1β, and H-7α and H-5α and H-8α in the NOESY spectrum (Figure 3). Thus, the structure of **3** was determined to be 3α-acetoxy-4α-hydroxy-

Table 3. NMR Spectral Data for **3**[a]

position	δ_C	δ_H	COSY	NOESY	HMBC
1 α	34.3 t[b]	1.42[c]	2		
β		1.23 dd (4.0, 12.3)	2		14
2	22.3 t	1.84[c]	1α, 1β	3β	10
3 β	77.4 d	4.78 t (2.7)	1α, 2	2, 15	
4	71.8 s				
5 α	48.9 d	1.58 dd (2.3, 12.8)	6α, 6β	6α, 7α	4
6 α	25.7 t	1.97 m	6β	5α, 6β	
β		1.20[c]	6α	6α	7, 11
7 α	41.4 d	2.25 m	6β, 8α	5α, 8α	
8 α	26.9 t	1.64[c]	8β	7α	10
β			1.39[c]	8α	
9 α	43.7 t	1.47 dt (2.9, 12.2)			7
β		1.30 dd (3.8, 12.2)			
10	34.0 s				
12	118.8 d	5.82 dd (1.3, 15.8)	11	11	8, 13
11	153.8 d	6.96 dd (6.6, 15.8)	7α, 12	12	7, 13
13	167.5 s				
14	18.2 q	0.92 s		2	1, 5, 9
15	21.3 q	1.15 s		3β	3, 4, 5
*C*OCH$_3$	170.7 s				
CO*C*H$_3$	21.4 q	2.13 s			*C*OCH$_3$
COO*C*H$_3$	51.5 q	3.73 s			13

[a] Spectra obtained at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR in $CDCl_3$. Figures in parentheses are coupling constants in Hz. [b] Carbon multiplicities were determined according to DEPT and HMQC spectra. [c] Multiplicity patterns were unclear due to signal overlapping.

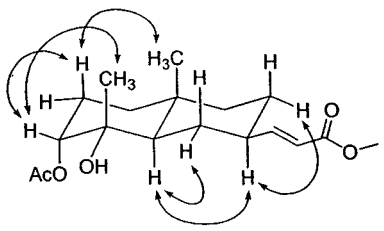

Figure 3. Selected ROESY correlations for **3**.

Table 4. Antiproliferation of Human Colon Cancer (Col2) Cells, Induction of Human Promyelocytic Leukemia (HL-60) Cell Differentiation, and Inhibition of DMBA-Induced Preneoplastic Lesion Formation Using the Mouse Mammary Organ Culture (MMOC) Model by Compounds **1**−**7**

compound	Col2 cell line[a]	HL-60 cell line[b]	MMOC[c]
1	>20	<10	NT[d]
2	>20	37.4	40.2
3	>20	<10	NT[d]
4	5.9	33.9	63.0
5	0.7	20.0	44.4
6	1.7	<10	44.4
7	18.9	32.4	48.7

[a] IC_{50} (half-maximal inhibitory concentration) values in μg/mL. [b] Activity denotes percentage of cells differentiated when treated with test compound (4 μg/mL), as indicated by nonspecific esterase determinations. [c] Percent inhibition was calculated in comparison with a DMBA (carcinogen) control. Based on historical controls, samples are classified as active if preneoplastic lesions are reduced to >60% at 10 μg/mL. [d] Not tested.

4β,10β-dimethyl-7β-(methyl 1*E*-propenoate)-*trans*-decalin (3α-acetoxydiversifolol).

The 11 isolates obtained in this investigation from the aerial parts of *T. diversifolia* were evaluated for their potential as chemopreventive agents by assessing antiproliferative activity in human colon cancer (Col2) cells,[16] induction of human promyelocytic leukemia (HL-60) cell differentiation,[17] and inhibition of DMBA-induced lesions in mouse mammary organ culture,[18] respectively. The results summarized in Table 4 illustrate that compounds **5** and **6** were active in the antiproliferation sulforhodamine B assay (IC_{50} ≤5 μg/mL), and compounds **2**, **4**, and **7** showed mild activity in the nonspecific esterase assay using the HL-60 cell differentiation model (>30% of cell differentiation at 4 μg/mL). The known compounds 3α-acetoxy-4α-hydroxy-11(13)-eudesmen-12-oic acid methyl ester, 17,20-dihydroxygeranylnerol, tagitinin A, and tirotundin were inactive in both of these in vitro bioassays. On the basis of their activities in the two in vitro tests, compounds **2** and **4**−**7** were selected for evaluation in the mouse mammary organ culture assay, and **4** exhibited significant inhibitory activity (63.0% at 10 μg/mL) and will be considered for further biological evaluation as a potential cancer chemopreventive agent. In contrast, compounds **2** and **5**−**7** were not regarded as active in the mouse mammary organ culture assay, since they did not meet the threshold value for inhibitory activity (>60% at 10 μg/mL).

Experimental Section

General Experimental Procedures. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. UV spectra were measured on a Beckman DU-7 spectrometer and IR spectra on an ATI Mattson FT-IR spectrometer. NMR spectra were recorded on Bruker DPX-300 or DRX-500 NMR spectrometers with TMS as internal standard. HRFABMS and LRFABMS were recorded on a VG 7070-HF mass spectrometer using a mixed matrix consisting of glycerol, thioglycerol, and 3-nitrobenzyl alcohol. Reversed-phase HPLC was carried out using a 19 × 300 mm, 6-μm Prep Nova-Pak C_{18} column (Waters, Milford, MA) at 8 mL/min and monitored at 210 nm. Normal-phase HPLC was carried out using a 19 × 300 mm, 10-μm Porasil Si gel column (Waters, Milford, MA) at 8 mL/min and monitored at 210 nm. For visualization of TLC plates, 10% (v/v) H_2SO_4 and phosphomolybolic acid reagent were used.

Plant Material. The aerial parts of *T. diversifolia* (Hemsley) A. Gray (Asteraceae) were collected at Villalba, Puerto Rico, in July 1999 and identified by F.A. A voucher specimen (accession no. 2218931) has been deposited at the Field Museum of Natural History, Chicago, IL.

Biological Assay for Antiproliferative Activity. This assay was carried out according to an established protocol.[16] In brief, a human colon cancer cell line (Col2) was cultured in MEME medium containing 10% nonessential amino acid solution (NEAA), 1× antibiotic-antimycotic, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. To perform this sulforhodamine B (SRB) assay, exponentially growing cells were added to 96-well microtiter plates containing test compounds dissolved in DMSO. Cells were allowed to grow at 37 °C, and, after 3 days, they were fixed with trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. The bound dye was solubilized in 0.1 M Tris, and the absorbance at 515 nm was measured. Percent growth was calculated from the following formula: % growth = $(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{day0}})/(\text{Absorbance}_{\text{DMSOcontrol}} - \text{Absorbance}_{\text{day0}}) \times 100$.

HL-60 Cell Differentiation Assay. Induction of cellular differentiation using HL-60 cells was assessed as previously described.[17] HL-60 (human promyelocytic leukemia) cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. Incubations were performed at 37 °C in a humidified atmosphere of 5% CO_2 in air. For testing, cells were diluted to 1.2×10^5 cells/mL and preincubated for 18 h. Samples dissolved in DMSO were prepared in 24-well plates, and HL-60 cells were then added, with the final DMSO concentration maintained at 0.1% v/v. After 4 days of incubation, the cells were analyzed to determine the percentage exhibiting functional and enzymatic markers of differentiated cells. Assays for α-naphthyl acetate esterase (nonspecific esterase, NSE) were performed using cytochemical kits from Sigma Chemical Co. (St. Louis, MO). Cell differentiation was assessed by microscopic examination of a minimum of 200 cells (in duplicate) for each experiment. NSE was detected primarily in monocytes, macrophages, and histocytes and was virtually absent in granulocytes.

Mouse Mammary Gland Organ Culture Assay. This assay was carried out according to an established protocol.[18] In brief, BALB/c female mice were pretreated for 9 days with 1 μg of estradiol and 1 mg of progesterone. On the tenth day, the mice were sacrificed and the second pair of thoracic mammary glands was dissected on silk and transferred to 60-mm culture dishes containing 5 mL of Waymouth's 752/1 MB medium supplemented with streptomycin, penicillin, and L-glutamine. The glands were incubated for 10 days (37 °C, 95% O_2 + 5% CO_2) in the presence of growth-promoting hormones (5 μg of insulin, 5 μg of prolactin, 1 μg of aldosterone, and 1 μg of hydrocortisone per mL of medium). Glands were exposed to 2 μg/mL DMBA between 72 and 96 h. After exposure, glands were rinsed and transferred to new dishes with fresh medium. The fully differentiated glands were then permitted to regress by withdrawing all hormones except insulin for 14 additional days. Test compounds were present in the medium during days 1−10 of culture; mammary glands were scored for incidence of lesions.

Extraction and Isolation. The milled air-dried aerial parts of *T. diversifolia* (720 g) were extracted with MeOH (4 L × 3) at room temperature, and the solution was evaporated in vacuo. On drying, this initial methanolic extract exhibited significant antiproliferative activity in the SRB assay performed with Col2 cells (IC_{50} 2.2 μg/mL), HL-60 cell differentiation induction activity (25.4% of differentiation at 4 μg/mL), and inhibitory activity (67.0% inhibition at a concentration of 10 μg/mL) in the mouse mammary gland organ culture assay. The dried MeOH extract (81 g) was resuspended in 10% H_2O in MeOH (500 mL) and partitioned with petroleum ether (500 mL × 3) to afford a petroleum ether-soluble residue (18.6 g). To the aqueous MeOH layer was added H_2O (200 mL), and this was then partitioned with EtOAc (700 mL × 2) to afford an EtOAc-soluble extract (23 g), which was followed by partitioning with a 1% aqueous solution of NaCl. The EtOAc-soluble extract exhibited significant antiproliferative activity (IC_{50} 1.3 μg/mL) and HL-60 cell differentiation induction activity (70% of differentiation at 4 μg/mL), while both the petroleum ether-soluble and aqueous-soluble extracts were

inactive (IC_{50} >20 μg/mL for the SRB assay; <10% of differentiation at 4 μg/mL for the HL-60 cell differentiation assay).

The EtOAc extract (23 g) was subjected to Si gel column chromatography and eluted with increasing concentrations of MeOH in $CHCl_3$ to give eight fractions. A portion of first fraction (3.0 g) was subjected to Si gel column chromatography eluted with $CHCl_3$−acetone in a step-gradient mode to afford **5** (1.0 g, eluted with $CHCl_3$−acetone, 10:1) and a fraction (1.1 g, eluted with $CHCl_3$−acetone, 50:1) that afforded seven further fractions (i−vii, SiO_2, *n*-hexanes−EtOAc, 20:1 to 4:1). The known compound, 3α-acetoxy-4α-hydroxy-11(13)-eudesmen-12-oic acid methyl ester (17 mg), was purified by normal-phase HPLC (*n*-hexane−2-propanol−MeOH, 7.4:0.3:0.3; t_R 22.3 min) from fraction iv (45 mg, SiO_2, *n*-hexanes−EtOAc, 8:1). Similarly, **3** (4 mg) and tirotundin (6 mg) were obtained from fraction vi (60 mg, SiO_2, *n*-hexanes−EtOAc, 6:1), followed by purification with normal-phase HPLC (*n*-hexane−2-propanol−MeOH, 7.2:0.4:0.4; t_R 14.0 and 23.4 min, respectively). In turn, the second fraction (3.6 g) was also fractionated (SiO_2, stepwise, $CHCl_3$−acetone) to give a fraction (800 mg, eluted with $CHCl_3$−acetone, 5:1) that was repeatedly subjected to Si gel column chromatography (stepwise, $CHCl_3$−acetone) to afford **5** (126 mg, eluted with $CHCl_3$−acetone, 8:1) and three further fractions (viii−x, eluted with $CHCl_3$−acetone from 7:1 to 5:1). Compounds **1** (5.6 mg) and **6** (9.0 mg) were purified from fraction viii (35 mg) by reversed-phase HPLC (MeOH−H_2O, 3.6:4.4; t_R 20.2 and 13.3 min, respectively). Similarly, compounds **4** (25 mg) and **7** (45 mg) were obtained from fraction ix (94 mg) by reversed-phase HPLC (CH_3CN−H_2O, 3.6:4.4; t_R 10.0 and 6.7 min, respectively), and **2** (15 mg) and tagitinin A (34 mg) from fraction x (65 mg) by reversed-phase HPLC (CH_3CN−H_2O, 3.0:5.0; t_R 13.5 and 11.1 min, respectively). Finally, 17,20-dihydroxygeranylnerol (60 mg) was obtained from the fourth fraction (1.3 g), after further fractionation (SiO_2, $CHCl_3$−acetone, 5:1), followed by purification with normal-phase HPLC (*n*-hexane−2-propanol−MeOH, 6.4:0.8:0.8; t_R 14.0 min).

(2*R*)-Dihydroxy-3,10-epoxy-8-isobutyloxygermacra-11-(13)-en-6,12-olide (2α-hydroxytirotundin, 1): light yellowish solid; $[\alpha]^{25}_D$ −78.0° (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ϵ) 211 (3.92) nm; IR (dried film) ν_{max} 3480, 3452, 1756, 1734, 1658, 1459, 1260, 1155 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRFABMS *m*/*z* 369 $[M + H]^+$; HRFABMS *m*/*z* 369.1900 $[M + H]^+$ (calcd for $C_{19}H_{29}O_7$, 369.1913).

(1*R*,3*S*,4*S*)-3-Acetoxy-1,4-dihydroxy-8-isobutyloxy-eudesm-11(13)-en-6,12-olide (tithofolinolide, 2): colorless gel; $[\alpha]^{25}_D$ −33.5° (*c* 0.47, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (3.94) nm; IR (dried film) ν_{max} 3489, 1772, 1730, 1468, 1384, 1247, 1153 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; LRFABMS *m*/*z* 411 $[M + H]^+$; HRFABMS *m*/*z* 411.2043 $[M + H]^+$ (calcd for $C_{21}H_{31}O_8$, 411.2019).

3α-Acetoxy-4α-hydroxy-4*β*,10*β*-dimethyl-7*β*-(methyl 1*E*-propenoate)-*trans*-decalin (3α-acetoxydiversifolol, 3): colorless gel; $[\alpha]^{25}_D$ −71.8° (*c* 0.071, MeOH); UV (MeOH) λ_{max} (log ϵ) 211 (3.98) nm; IR (dried film) ν_{max} 3512, 1723, 1658, 1440, 1374, 1243 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; LRFABMS *m*/*z* 347 $[M + Na]^+$; HRFABMS *m*/*z* 347.1836 $[M + Na]^+$ (calcd for $C_{18}H_{28}O_5Na$, 347.1834).

Preparation of (*S*)- and (*R*)-MTPA Ester Derivatives of 1. A mixture of 0.5 mg of **1**, 0.2 mg of 4-(dimethylamino)-pyridine, and 10 mg of (*R*)-(−)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride in 400 μL of anhydrous pyridine was kept standing at room temperature for 8 h and then passed through a disposable pipet (0.6 × 5 cm) packed with Si gel and eluted with 4 mL of $CHCl_3$−acetone (10:1). The solvent was removed in vacuo, to obtain the *S*-Mosher ester, **1s**. Treatment of 0.5 mg of **1** with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride, as described above, yielded the *R*-Mosher ester, **1r**. ^1H NMR data for **1s** and **1r** are presented in Table 1.

Preparation of (*S*)- and (*R*)-MTPA Ester Derivatives of 2. Similar to the above procedure described for compound **1**, treatment of 0.5 mg each of **2** with (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride yielded the *S*-Mosher ester, **2s**, and the *R*-Mosher ester, **2r**, respectively. ^1H NMR data for **2s** and **2r** are presented in Table 2.

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