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Antiproliferative Constituents of the Roots of Ethiopian *Podocarpus falcatus* and Structure Revision of 2α-Hydroxynagilactone F and Nagilactone I

Ermias Mekuria Addo,[†,‡] Hee-Byung Chai,[†] Ariaya Hymete,[‡] Mariamawit Yonathan Yeshak,[‡] Carla Slebodnick,[§] David G. I. Kingston,[§] and L. Harinantenaina Rakotondraibe*[,†]

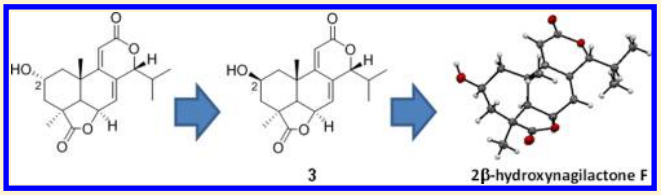
[†]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, United States

[‡]Department of Pharmaceutical Chemistry and Pharmcognosy, School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

[§]Department of Chemistry and Virginia Tech Center for Drug Discovery, M/C 0212, Virginia Tech, Blacksburg, Virginia 24061, United States

Supporting Information

ABSTRACT: Bioassay-guided fractionation using the human colorectal adenocarcinoma (HT-29) cell line of the methanol extract of dried roots of *Podocarpus falcatus* led to the isolation of two new type C nagilactones, 16-hydroxynagilactone F (**1**) and 2β,16-dihydroxynagilactone F (**2**), and the new totarane-type bisditerpenoid 7β-hydroxymacrophyllic acid (**4**), along with the seven known compounds 2β-hydroxynagilactone F (**3**), macrophyllic acid (**5**), nagilactone D (**6**), 15-hydroxynagilactone D (**7**), nagilactone I (**8**), inumakiol D (**9**), and ponasterone A (**10**). The structures of the new compounds were determined by 1D and 2D NMR, HRESIMS, UV, and IR and by comparison with the reported spectroscopic data of their congeners. The orientation of the C-2 hydroxy group of **3** and **8** was revised to be β based on evidence from detailed analysis of 1D and 2D NMR data and single-crystal X-ray diffraction studies. Among the isolated compounds, the nagilactones, including the new dilactones 16-hydroxynagilactone F (**1**) and 2β,16-dihydroxynagilactone F (**2**), were the most active (IC_{50} 0.3−5.1 μM range) against the HT-29 cell line, whereas the bisditerpenoids (**4** and **5**) and the other known compounds **9** and **10** were inactive. The presence of the bioactive nagilactones in *P. falcatus* supports its traditional use.



Cancer has claimed the lives of millions of people around the world. In 2012 alone, 14.1 million new cases have been recorded, and this raised the number of people living with cancer to 32.6 million at that time.[1] The nonselective toxicity[2] of current anticancer drugs and the increasing resistance of certain types of cancer are the major problems in the treatment of cancer.[3,4] The number of new cases is expected to rise to 22 million within the next two decades if the current pharmaceutical arsenal to treat cancer has not improved.[5] Discovering new anticancer drugs with new and safe modes of action is thus urgently needed.

Natural products are well-known contributors of anticancer drugs.[6,7] Among higher plants, species of *Taxus*,[8,9] *Vinca* (*Catharanthus*),[10] *Podophyllum*,[11,12] and *Camptotheca*[13] are examples of the genera of known sources of clinically used anticancer drugs. Investigation of African traditional medicines, including medicinal plants, is one of the approaches used in our laboratory to discover new anticancer compounds. Our ongoing screening of natural product extracts including medicinal plant extracts from Ethiopia and Madagascar led to the identification of an antiproliferative methanol extract (ED_{50} 10 μg/mL) of an Ethiopian medicinal plant identified as *Podocarpus falcatus* (Thunb.) R.Br. ex Mirb. (Podocarpaceae) with the vernacular name *zigiba*.

Podocarpus is one of the largest genera of all conifers of the family Podocarpaceae. The genus *Podocarpus* contains 94 species distributed from south temperate zones through the tropical highlands, West Indies, and Japan.[14] Species from this genus have been reported to produce cytotoxic nor- and bisnor-diterpenoid dilactones generally known as nagilactones or podolactones.[15] In addition, totarane-type diterpenes such as totarol and their dimers (e.g., macrophyllic acid, **5**) have been also isolated from many species of *Podocarpus*, and they are considered to be chemical markers of the genus.[16] The unusual structures of nagilactones and their wide range of biological activities have drawn particular interest from chemists and pharmacologists working in the drug discovery area.[17] Nagilactones are dilactone-containing nor- and bisnor-diterpenoids with rings designated as A, B, and C similar to those found in abietane, totarane, and podocarpane diterpenoids (Figure 1). On the basis of the unsaturation patterns of B and C rings, nagilactones are classified into three major groups: type

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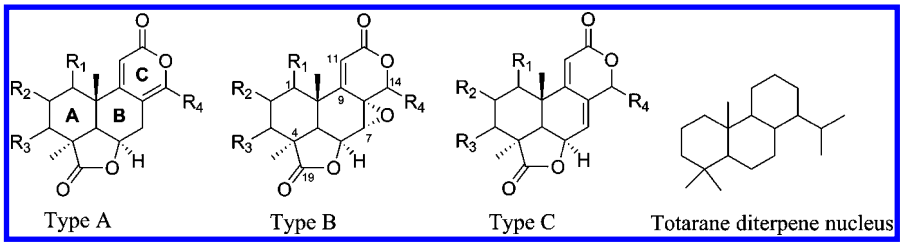
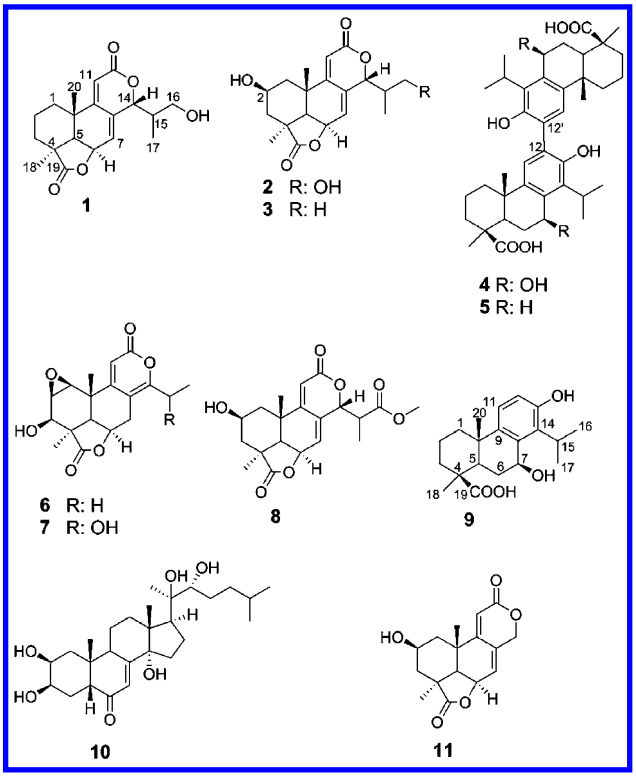


Figure 1. Types of nagilactones and totarane diterpene nucleus.

A, which are those with α-pyrone [8(14),9(11)-dienolide], type B with 7α,8α-epoxy-9(11)-enolide, and type C with 7(8),9(11)-dienolide moieties.[15,18]

P. falcatus (Thunb.) R.Br. ex Mirb. is an evergreen, dioecious, medium- to large-sized tree up to 60 m tall widely distributed in Ethiopia, Kenya, Tanzania, Mozambique, South Africa, and Madagascar.[14,19] Besides other commercial and ecological importance, ethnobotanical reports from some parts of Ethiopia indicate that preparations from the roots of this plant are used as "anticancer" remedies.[20,21] The antioxidant properties, COX and tyrosinase inhibitory activities, and cytotoxicity of extracts from leaves and stems of the plant have also been reported.[22,23] The wood of *P. falcatus* has been shown to contain macrophyllic acid (**5**) and totarol.[24] The present investigation deals with the isolation and characterization of the two new type C nagilactones 16-hydroxynagilactone F (**1**) and 2β,16-dihydroxynagilactone F (**2**) and a new bisditerpenoid, 7β-hydroxymacrophyllic acid (**4**), along with four known nagilactones (**3**, **6**, **7**, and **8**), the totarol dimer macrophyllic acid (**5**), the totarane-type diterpenoid inumakiol D (**9**), and the phytoecdysteroid ponasterone A (**10**). In this study, the structures of **3** and **8**, which were identified as 2α-hydroxynagilactone F[25,26] and the methyl ester of 2α-hydroxynagilactone F 16-oic acid (nagilactone I),[27] respectively, are also revised based on NMR spectroscopic data and X-ray diffraction analyses.



RESULTS AND DISCUSSION

The antiproliferative methanolic extract of air-dried roots of *P. falcatus* was fractionated with hexanes, EtOAc, and H_2O. Bioassay on HT-29 showed that the EtOAc fraction was the most active (ED_{50} of 3 μg/mL). Subsequent liquid–liquid partition and fractionations on Sephadex LH-20 and column chromatography resulted in two fractions with improved activities of ED_{50} 0.4 μg/mL. Reversed-phase C_{18} HPLC of each active fraction resulted in the isolation of the two new type C nagilactones, 16-hydroxynagilactone F (**1**) and 2β,16-dihydroxynagilactone F (**2**), and a bisditerpenoid, 7β-hydroxymacrophyllic acid (**4**), along with seven known compounds, identified as 2β-hydroxynagilactone F (**3**), macrophyllic acid (**5**), nagilactone D (**6**), 15-hydroxynagilactone D (**7**), nagilactone I (**8**), inumakiol D (**9**), and ponasterone A (**10**). The structure of **7** has been reported without ^{13}C NMR data,[28] and in the present report we included the full NMR data of **7** (Tables 3 and 4).

Structure Revision of 3 and 8. The C-2 hydroxy group of **3** and **8** was reported to be α-oriented.[25−27] The ^{1}H and ^{13}C NMR spectroscopic data as well as the optical rotations of the newly isolated compounds **3** and **8** are superposable with reported data (Tables 1 and 2). Minimization of the energy of the two structures of **3** with 2α and 2β hydroxy groups using the MM2 program in the ChemBioDrawUltra software (version 14.0.0.117, CambridgeSoft Corporation) showed that both compounds have a boat conformation of the ring A. This observation was different from the reported data suggesting a chair conformation of the parent compounds.[29,30] Moreover, it is noteworthy that comparison of our NMR data of **3** with the data of reported compounds[25−27] led to the conclusion that the assignments of the ^{13}C NMR chemical shifts of C-18 and C-20 methyl groups of compound **3** in ref 25 should be interchanged.

Analysis of the splitting patterns of the H-2 resonance in the ^{1}H NMR spectrum and the 2D-NOESY of both compounds showed that the orientation of the hydroxy group should be β. The dddd (J = 12.9, 10, 7.2, 5.1 Hz) splitting of H-2 (δ 4.08) for compound **3** showed that two large couplings were present, and this was also reported by the previous authors (Table 1). These coupling patterns were possible only if the H-2 hydroxy group is β-oriented (Figure 2). In addition, H-2 of compounds **3** and **8** exhibited NOE cross-peaks with H-3α and H-18. Both H-3α and H-18 also correlated with H-5 and H-6, while H-1β and H-3β correlated with the CH_3-20 singlet.

To confirm these assignments, compounds **3** and **8** were crystallized and subjected to X-ray crystallographic analysis. These studies confirmed the 2β-OH orientation for both compounds (Figure 3). The ^{1}H and ^{13}C NMR data arising from the A ring are in agreement with the data reported for the tetranor-diterpenoid wentilactone B (**11**), which has been

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Table 1. ^{1}H NMR (400 MHz) Data for Compounds 3 and 8

position		Kubo et al., 1991[25]	Bloor et al., 1991[26]	present data	Ying et al., 1990[27]	present data	
		3[a]	3[b]	3[c]	8[a]	8[a]	8[b]
1	a	2.48 dd (13.7, 9.0)	2.24 t (13)	2.42 dd (13.4, 10)	2.47 dd (13.3, 9.6)	2.50 dd (13.6, 9)	2.4 dd (13.6, 9.2)
	b	1.84 dd (13.7, 6.8)	1.91 (obsc.)	1.53 dd (13.4, 7.2)	2.17 dd (13.3, 4.9)	2.20 dd (13.6, 7.2)	1.58 dd (13.6, 7.2)
2		4.31 m ($W_{1/2}$ 26 Hz)	4.11 m	4.08 dddd (12.9, 10, 7.2, 5.1)	4.28 m ($W_{1/2}$ 28 Hz)	4.31 dddd (12.6, 9, 7.2, 5.1)	4.1 br s
3	a	2.56 dd (13.7, 12.8)	2.45 dd (14, 9)	2.11 br t (13.4)	2.54 dd (13.3, 12.4)	2.57 t (13.2)	2.20 br t (13.5)
	b	2.19 dd (13.7, 5.1)	1.59 dd (14, 7)	1.84 dd (13.4, 5.1)	1.85 d (12.4)	2.20 dd (13.7, 5.1)	1.92 dd (13.5, 5.2)
4							
5		1.82 d (5.1)	1.95 d (5)	2.18 d (5)	1.83 d (5.6)	1.84 d (5)	1.92 d (5)
6		5.14 dd (5.1, 2.1)	5.08 ddd (5, 5, 1.5)	5.23 ddd (5, 5, 1.7)	5.16 t-like	5.19 ddd (5, 5, 1.6)	5.00 ddd (5, 5, 1.7)
7		6.27 br d (2.1)	6.20 ddd (5, 1.5)	6.35 ddd (5, 1.7, 1.6)	6.43 d (2.1)	6.46 ddd (5, 1.9, 1.7)	6.13 ddd (5, 1.8, 1.7)
8							
9							
10							
11		6.05 s	5.83 d (1.6)	5.83 d (1.7)	6.09 s	6.12 d (1.7)	5.82 d (1.7)
12							
13							
14		4.86 br s	4.87 d	5.01 ddd (2.3, 1.7, 1.6)	5.32 br s	5.34 ddd (4.1, 1.9, 1.6)	5.21 ddd (4, 1.8, 1.7)
15		2.22 dqq (6.8, 6.8, 2.1)	2.25 m	2.4 sd (6.8, 2.3)	3.39 dq (6.8, 3.9)	3.42 qd (7.1, 4.1)	3.16 qd (7.2, 4)
16		0.98 d (6.8)	0.98 d (6)	1.18 d (6.8)			
17		1.13 d (6.8)	1.20 d (6)	0.97 d (6.8)	1.46 d (6.8)	1.48 d (7.1)	1.38 d (7.2)
18		1.39 s	1.42 s	1.40 s	1.30 s	1.38 s	1.4 s
19							
20		1.29 s	1.28 s	1.24 s	1.36 s	1.32 s	
$-OCH_3$					3.63 s	3.65 s	3.70 s

[a]Data collected in pyridine-d_5. [b]Data collected in chloroform-d. [c]Data collected in methanol-d_4.

Table 2. ^{13}C NMR (100 MHz) Data for Compounds 3 and 8

position	Kubo et al., 1991[25]	Bloor et al., 1991[26]	present data	Ying et al., 1990[27]	present data	
	3[a]	3[b]	3[c]	8[a]	8[a,d]	8[b]
1	41.2	40.2	41.3, CH_2	41.0	41.5, CH_2	40.4, CH_2
2	63.9	64.7	65.3, CH	63.9	64.3, CH	64.8, CH
3	37.8	36.5	37.7, CH_2	37.6	38.1, CH_2	36.7, CH_2
4	42.8	42.4	44.0, C	42.8	43.2, C	42.5, C
5	45.5	45.7	46.6, CH	45.3	45.7, CH	45.8, CH
6	72.4	72.0	74.1, CH	72.4	72.8, CH	72.0, CH
7	122.4	121.6	123.9, CH	122.5	122.7, CH	121.9, CH
8	133.8	133.8	134.8, C	133.2	133.7, C	132.8, C
9	159.0	158.3	161.3, C	158.8	159.2, C	158.3, C
10	36.1	35.9	37.3, C	36.2	36.6, C	36.2, C
11	113.1	112.8	113.2, CH	113.1	113.6, CH	112.9, CH
12	164.1	165.0	166.8, C	163.5	163.8, C	163.2, C
13						
14	83.0	83.0	85, CH	80.0	80.4, CH	79.5, CH
15	29.8	29.7	31.4, CH	42.4	42.8, CH	42.8, CH
16	16.3	15.1	20.1, CH_3	172.6	173.0, C	172.3, C
17	19.7	19.6	15.6, CH_3	13.8	14.1, CH_3	12.8, CH_3
18	23.4	27.9	28.7, CH_3	28.0	28.4, CH_3	28.2, CH_3
19	181.6	180.6	183.8, C	181.6	181.9, C	180.8, C
20	27.8	23.4	23.7, CH_3	23.3	23.8, CH_3	23.6, CH_3
$-OCH_3$				51.9	52.3, CH_3	52.5, CH_3

[a]Data collected in pyridine-d_5. [b]Data collected in chloroform-d. [c]Data collected in methanol-d_4. [d]Part of the assignments of the carbon multiplicities were performed based on their similarities with the reported data.[25−27]

isolated from the endophytic fungus *Aspergillus wentii* EN-48.[31,32]

From these evidences, the structure of **3** should be revised to 2β-hydroxynagilactone F and the orientation of the C-2

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Table 3. ^{1}H NMR (400 MHz) Data for Compounds 1, 2, 4, and 7

position		1[a]	2[a]	4[b,e]	4[c,e]	7[d]
1	a	1.69 m (overlapped)[g]	2.42 dd (13.5, 9.1)	2.43 m (overlapped)[g]	1.32 (overlapped)[g]	3.57 d (4.2)
	b	1.79 m (overlapped)[g]	1.54 dd (13.5, 7.2)	1.45 m (overlapped)[g]	2.14 (overlapped)[g]	
2	a	1.75 m (overlapped)[g]	4.06 dddd (12.6, 9.1, 7.2, 5.1)	2.38 m (overlapped)[g]	1.52 br d (11.4)	3.48 dd (6, 4.2)
	b	1.84 m (overlapped)[g]		1.58 m (overlapped)[g]	1.89 (overlapped)[g]	
3	a	1.55 m	2.11 br t (13.5)	2.58 br t (10.5)	1.04 (overlapped)[g]	4.45 dd (6, 2.2)
	b	2.18 m	1.84 dd (13.5, 5.1)	1.18 br t (11.6)	2.11 (overlapped)[g]	
4						
5		2.08 d (4.8)	2.11 d (5)	2.58 (overlapped)	1.96 (overlapped)[g]	1.88 d (6.4)
6	a	5.22 ddd (4.8, 4.8, 1.7)	5.22 ddd (5,5, 1.6)	2.98 br d (12.6)	2.00 (overlapped)[g]	4.94 dt (9.8, 6.4, 6.4)
	b			2.85 br t (12.6)	2.15(overlapped)[g]	
7	a	6.42 ddd (4.8, 1.9, 1.9)	6.42 ddd (5, 1.8, 1.6)	5.56 br s	4.78 br s	3.54 dd (16.8, 9.8)
	b					2.74 dd (16.8, 6.4)
8						
9						
10						
11		5.79 d (1.9)	5.82 d (1.6)	7.50 s	6.91 s	6.37 s
12						
13						
14		5.09 ddd (2.0, 1.9,1.7)	5.09 ddd (2.0, 1.8, 1.6)			
15		2.39 m	2.37 m	4.29 sep (6.6)	3.56[f]	4.87 dq (9.5, 6.6)
16	a	3.76 dd (11.0, 4.4)	3.74 dd (11.0, 4.5)	1.88 d (6.6)	1.34 d (7.1)	1.52 d (6.6)
	b	3.47 dd (11.0, 8.0)	3.46 dd (11.0, 8.0)			
17		1.22 d (6.8)	1.23 d (6.8)	1.77 br s	1.32 d (7.4)	
18		1.33 s	1.40 s	1.59 s	1.18 s	1.43 s
19						
20		1.16 s	1.21 s	1.42 br s	0.98 s	1.28 s
3-OH						3.4 d (2.2)
15-OH						2.26 d (9.5)

[a]Measured in methanol-d_4. [b]Measured in pyridine-d_5. [c]Measured in DMSO-d_6. [d]Measured in chloroform-d. [e]Assignment of the monomer. [f]Overlapped with the water signal. [g]The overlapped signals were assigned with accurate axis calibration of ^{1}H−^{1}H COSY and HSQC.

Table 4. ^{13}C NMR (100 MHz) Data for Compounds 1, 2, 4, and 7

position	1[a]	2[a]	4[b,g]	4[c,g]	7[d]
1	31.2, CH_2	41.3, CH_2	40.7, CH_2	39.5, CH_2[f]	57.2, CH
2	18.7, CH_2	65.3, CH	21.3, CH_2	20.3, CH_2	50.7, CH
3	29.1, CH_2	37.7, CH_2	38.8, CH_2	37.6, CH_2	67.6, CH
4	44.4, C	44.0, C	44.2, C	42.9, C	48.8, C
5	48.5, CH	46.6, CH	45.9, CH	44.5, CH	50.4, CH
6	73.9, CH	74.2, CH	33.2, CH_2	31.6, CH_2	73.2, CH
7	124.0, CH	123.9, CH	65.6, CH	64.0, CH	24.9, CH_2
8	135.0, C	134.6, C	137.5, C	134.6, C	108.4, C
9	161.9, C	161.3, C	142.6, C	140.9, C	162.5, C[e]
10	36.6, C	37.4, C	39.7, C	38.5, C	37.9, C
11	112.5, CH	113.2, CH	127.6, CH	126.0, CH	106.6, CH
12	166.6, C	166.5, C	130.9, C	130.6, C	162.6, C[e]
13			152.3, C	151.5, C	
14	83.5, CH	83.5, CH	136.6, C	136.0, C	160.7, C[e]
15	39.3, CH	39.5, CH	29.2, CH	27.7, CH	64.7, CH
16	63.5, CH_2	63.4, CH_2	21.8, CH_3	21.4, CH_3	22.0, CH_3
17	15.2, CH_3	15.2, CH_3	22.0, CH_3	21.4, CH_3	
18	24.6, CH_3	28.8, CH_3	29.8, CH_3	28.6, CH_3	25.9, CH_3
19	183.8, C	183.8, C	180.6, C	179.6, C	177.9, C[e]
20	25.7, CH_3	23.7, CH_3	23.7, CH_3	22.9, CH_3	17.7, CH_3

[a]Measured in methanol-d_4. [b]Measured in pyridine-d_5. [c]Measured in DMSO-d_6. [d]Measured in chloroform-d. [e]Signal assignments were from HMBC. [f]Overlapped with the solvent signal. [g]Assignment of the monomer.

hydroxy group of nagilactone I (8), methyl 2β-hydroxynagilactone F-16-oate, should be β as depicted.

The X-ray crystal structure of the related compound 6, a bisnor-diterpenoid that is consistent with the reported structure,[33] is also given in the Supporting Information.

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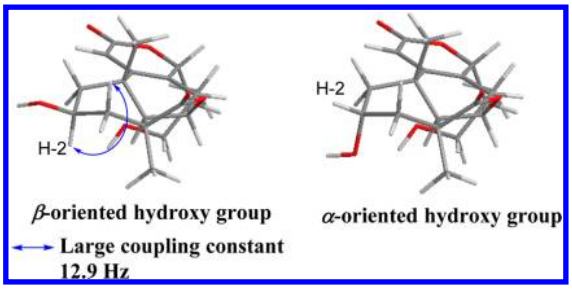

Figure 2. Stable conformations of the 2α- and 2β-hydroxynagilactone F after MM2 energy minimization.

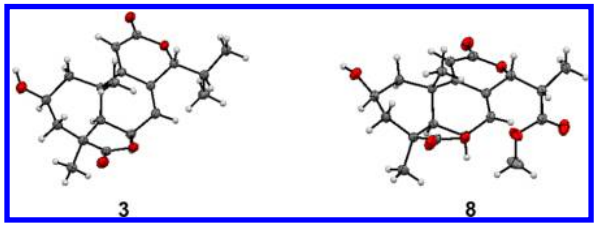

Figure 3. X-ray ORTEP drawing of the crystal structures of **3** and **8**.

Structure Elucidation of the New Compounds 1, 2, and 4. Compound **1** exhibited a sodiated pseudomolecular ion peak $[M + Na]^+$ at m/z 355.1500 (calcd 355.1516), which in conjunction with the ^{13}C NMR data indicated a molecular formula of $C_{19}H_{24}O_5$, identical to that of **3**. The UV spectrum showed an absorption band at λ_{max} of 259 nm, and the IR stretching at 1762 cm^{-1} (γ-lactone), 1701 cm^{-1} (δ-lactone), and 1597 cm^{-1} (weak; C═C conjugated with C═O) suggested that **1** is a type C nagilactone.[16] The ^1H NMR spectroscopic data of **1** (Table 3) were similar to those of **3** except for the absence of an H-2α signal (δ 4.08, dddd), the methyl doublet at δ 0.97 of **3**, and the presence of two doublet of doublets at δ 3.76 (dd, J = 11, 4.4 Hz) and 3.47 (dd, J = 11, 8 Hz) in addition to multiplets corresponding to three methylenes at δ 2.22−2.15 and 1.84−1.52. The ^{13}C NMR (Table 4) and HSQC revealed the presence of 19 carbon resonances with two carbonyl carbons indicative of γ-lactone (δ 183.8, C-19) and δ-lactone (δ 166.6, C-12) moieties, four olefinic carbons, two of which were monosubtituted (δ 112.5, C-11 and δ 124.0, C-7), two oxymethines (δ 73.9, C-6 and δ 83.5, C-14), an oxymethylene (δ 63.5, C-16), two quaternary carbons (δ 44.4, C-4 and δ 36.6, C-10), three methylenes (δ 31.2, C-1; δ 18.7, C-2, and δ 29.1, C-3), two methines (δ 48.5, C-5 and δ 39.3, C-15), and three methyl carbons at δ 15.2, 24.6, and 25.7 ascribable to C-17, C-18, and C-20, respectively.

Comparison of the ^1H and ^{13}C NMR of **1** with those of **3** revealed that the difference between the two compounds was the oxidation states of the ring A and one of the isopropyl methyls at C-15. Two-dimensional NMR including HSQC, $^1H-^1H$ COSY, HMBC, and NOESY were performed in order to determine the planar structure of **1**. The $^1H-^1H$ COSY correlations observed from H-1 to H-3 (Figure 4) demonstrated the absence of a C-2 hydroxy group in **1**. The long-range cross-peaks from the two pairs of diastereotopic oxygen-bearing methylene doublet of doublets at δ 3.47 and 3.76 to C-14, C-15, and C-17 permitted the location of the hydroxymethylene group at C-15 (Figure 4). This is in accordance with the absence in **1** of one isopropyl methyl doublet of **3** (δ 0.97, d, J = 6.8 Hz). The HMBC correlations from the oxygen-bearing methylene protons with C-15, C-17, and C-14 together

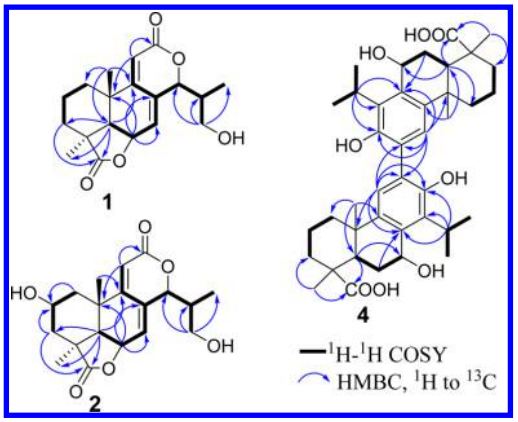

Figure 4. Key HMBC and $^1H-^1H$ COSY correlations of the new compounds **1**, **2**, and **4**.

with that of H-17 with C-16 confirmed that one of the isopropyl methyls (C-16) was substituted (Figure 4). The presence of a γ-lactone moiety was evidenced by the HMBC correlations from H-5 (δ 2.08, d, J = 4.8 Hz) to C-1, C-3, C-9, C-10, C-18, and C-19, from CH_3-18 to C-3, C-4, C-5, and C-19 (δ 183.8), and from H-20 to C-1, C-5, C-9, and C-10, while H-11 showed a correlation to the δ-lactone carbonyl at C-12 (δ 166.6). The relative configuration of **1** was deduced from the data observed in the 2D-NOESY spectrum (Figure 5). The orientation of the other substituents of the stereogenic carbons of **1** was deduced to be the same as those in **3** by analysis of the NOE cross-peaks observed in the NOESY spectrum (Figure 5). From the above data the structure of **1** was deduced to be 16-hydroxynagilactone F.

The molecular formula of **2** was determined to be $C_{19}H_{24}O_6$ from the ^{13}C NMR data and an HRESIMS pseudomolecular ion peak at m/z 371.1460 (calcd for $[M + Na]^+$ 371.1465). The UV absorption at λ_{max} 261 nm along with the IR stretching absorption at 1764 cm^{-1} (γ-lactone), 1698 cm^{-1} (δ-lactone), and 1600 cm^{-1} (C═C conjugated with a carbonyl) suggested that **2** also belonged to type C (7(8),9(11)-dienolide) norditerpene dilactones.[16] The ^1H NMR spectroscopic data of **2** (Table 3) were similar to those of **3** except for the absence of one of the isopropyl methyl doublets (δ 0.97) in **2** and the appearance of two doublet of doublets signals of hydroxymethylene protons at δ 3.74 and 3.46 ppm, which were identical to those of **1**. The ^{13}C NMR (Table 4) and HSQC data also indicated that **2** had a total of 19 resonances with two disubstituted olefinic carbons (δ 161.3, C-9 and 134.6, C-8), two monosubstituted olefinic carbons (δ 123.9, C-7 and 113.2, C-11), three oxymethine carbons (δ 83.5, C-14; 74.2, C-6; and 65.3, C-2), an oxymethylene carbon (δ 63.4, C-16), two methylene carbons (δ 41.3, C-1 and 37.7, C-3), two methine carbons (δ 46.6, C-5 and 39.5, C-15), three methyl carbons (δ 15.2, C-17; 23.7, C-20; and 28.8, C-18) and two quaternary carbons (44.0, C-4 and 37.4, C-10). The remaining two signals at δ 183.8 (C-19) and 166.5 (C-12) were typical of the carbonyls of γ-lactone and conjugated δ-lactone moieties, respectively, characteristic of the type C nagilactones.

Comparison of the ^1H and ^{13}C NMR data of **2** with those of **3** revealed that the only difference was the presence of a C-15 hydroxymethylene group in **2** instead of a C-15 methyl group in **3**. This was confirmed by the HMBC correlations from H-16 to C-15, C-17, and C-14 (Figure 4). The location of the hydroxy group at C-2 was evidenced by the presence of the spin

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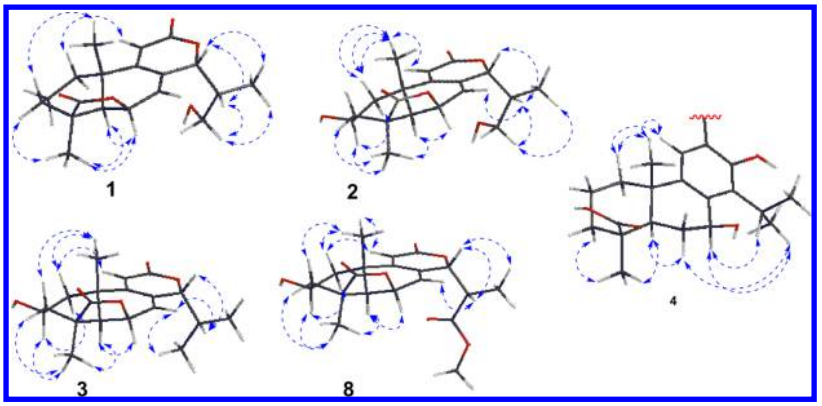

Figure 5. Key NOESY correlations of compounds **1**−**4** and **8**.

network H-1 (δ 2.42 and 1.54)−H-2 (δ 4.06)−H-3 (δ 2.11 and 1.84) in the COSY spectrum. The locations of the C-7 and C-9 double bonds were substantiated by the COSY spin system, H-5 (δ 2.11)−H-6 (δ 5.22)−H-7 (δ 6.42)−H-11(δ 5.82), and also from the HMBC correlations from H-6 to C-7 (δ 123.9) and C-8 (δ 134.6) and from H-15 to C-8. The assignment of the two lactone rings was confirmed by comparison of the ^{13}C NMR data of **2** with those of **3** and interpretation of the HMBC spectroscopic data. The determination of the relative configuration of **2** was carried out as follows. The correlation between H-2 (δ 4.06) and the methyl signal at H-18 (δ 1.40, s) in the NOESY spectrum of **2** indicated that the C-2 hydroxy was β-oriented (Figure 5). This was confirmed not only by the splitting pattern of the H-2 resonance, which was similar to those in **3** and **8**, but also by the NOE correlation between H-3β and CH_3-20 (δ 1.21), which in turn correlated with H-1β. The 14*S* configuration was deduced by comparison of the ^{1}H and the ^{13}C NMR chemical shifts of C-14 of **2** with those of **1** and **3**. The key HMBC correlations in support of the structure elucidation of **2** are shown in Figure 4. Thus, the complete structure of **2** was assigned as 2β,16-dihydroxynagilactone F.

Examination of the UV spectroscopic data of **4** showed absorptions at λ_{max} 217, 251, and 291 nm, which are similar to those of the totarol dimer macrophyllic acid **5** (λ_{max} 220, 254, and 290 nm) originally isolated from the heartwood of *P. macrophyllus* D. Don.[34] The IR spectrum exhibited absorption bands and stretch frequencies at 3533, 3500−2400 (broad), 1694, 1614, 1467, 1259, and 1175 cm^{-1} ascribable to hydroxy, carboxylic acid OH, carbonyl, aromatic methine, and phenolic hydroxy functions.[34,35] The HRESIMS displayed a sodiated pseudomolecular ion peak $[M + Na]^+$ at *m*/*z* 685.3727 (calcd 685.3711), which in conjunction with the ^{13}C NMR data indicated a molecular formula of $C_{40}H_{54}O_8$, which had two more oxygen atoms than **5**. The difference between **4** and **5** was thus suggested to be the presence of two additional oxygen atoms in **4**.

The ^{13}C NMR (Table 4) and HSQC indicated 20 resonances for the monomeric units: a carboxylic acid carbonyl (δ 180.6), an oxygenated aromatic carbon (δ 152.3), four quaternary aromatic carbons (δ 142.6, 137.5, 136.6, 130.9), an aromatic methine carbon (δ 127.6), an oxymethine carbon (δ 65.6), two methine carbons (δ 45.9, 29.2), two quaternary carbons (δ 44.2, 39.7), four methylenes (δ_C 40.7, 38.8, 33.2, 21.3), and four methyl carbons (δ 29.8, 23.7, 22.0, 21.8) (Table 4). With the exception of the carbon resonance at δ 130.9, the ^{13}C NMR spectrum of **4** was similar to that of inumakiol D (**9**), a known totarane-type monomer isolated from the bark of *P. macrophyllus* D. Don[36] (vs 117.3 in **9**). Likewise, the ^{1}H NMR spectroscopic data of **4** were also close to those of **9** except for the absence of the two aromatic doublets of **9** (δ 6.64, d, J = 8.7 Hz and 6.97, d, J = 8.7 Hz) compared to the aromatic singlet (δ 7.50, s) in **4**. This, coupled with the HRESIMS data with two extra oxygen atoms in **4** and the similarity of the IR and UV spectroscopic data with **5**, indicated that **4** was a dimer of **9**.

Noteworthy, most of the proton and carbon signals in the ^{1}H and ^{13}C NMR spectra of **4** were broadened. To gain more information on the structure of **4**, the NMR spectroscopic data of **4** were measured in DMSO-d_6 (Tables 3 and 4). The assignments of all carbons and protons in **4** as well as the site of the dimerization of the two monomers were successfully achieved by the interpretation of the NMR data obtained from three solvents (methanol-d_4, pyridine-d_5, and DMSO-d_6). The assignment of the oxygen-bearing methine at δ 5.56 (δ_C 65.6) to be at C-7,7′ was determined by the presence of the spin network from H-5,5′ to H-7,7′ [δ 2.58 (H-5,5′), δ_H 2.98 and 2.85 (H-6ab,6′ab), and δ_H 5.56 (H-7,7′)] in the ^{1}H−^{1}H COSY spectrum. This was in accordance with the similarity of the carbon chemical shifts of C-5,5′, C-6,6′, and C-7,7′ (45.9, 33.2, and 65.6 ppm, respectively) of **4** to those reported for **9**. Apart from the solvent signals (pyridine-d_5), the aromatic region of the ^{1}H NMR spectrum of **4** displayed only one signal (δ 7.50, s), which was attributed to two aromatic methines of two equivalent pentasubstituted aromatic rings. The assignment of the two aromatic methines to be at H-11,11′ resulted from the observation of the NOESY correlation from the signal at δ 7.50 to H-1*a* (δ 2.43) and to the methyl singlet at δ 1.42 (CH_3-20,20′). The location of the isopropyl moiety at C-14,14′ of **4**, which confirmed its totarane nature, was further confirmed by the NOESY correlation from H-7 (δ 5.56, br s) to the isopropyl methine proton septet at δ 4.29 (H-15,15′, sept, J = 6.6 Hz). This was confirmed by the long-range cross-peak from the isopropyl methyl signals [δ 1.88 (H-16,16′) and 1.77 (H-1,17′)] and isopropyl methine [δ 29.2 (C-15,15′) and 136.6 (C-14,14′)]. The long-range correlation from both H-11,11′ and H-15,15′ to the oxygen-bearing aromatic carbon (δ_C 151.5) permitted the location of the hydroxy group at C-13,13′. This was also supported by the correlation of both protons (H-11,11′ and H-15,15′) with the quaternary aromatic carbon C-8. These data indicated that the second monomeric unit must be attached at C-12. The strong HMBC correlation observed between H-11,11′ and C-12,12′ confirmed the ^2J (H-11 to C-12 or H-11′ to C-12′) and ^3J (H-11 to C-12′ or H-11′ to C-12) couplings. Therefore, the structure of **4**, named 7β-hydroxymacrophyllic acid, was elucidated as shown.

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Besides the new compounds, the structure of the known compounds were determined by comparison of their observed and reported spectroscopic and physical data: macrophyllic acid (**5**),[37] nagilactone D (**6**),[33] 15-hydroxynagilactone D (**7**),[28] inumakiol D (**9**),[36] and ponasterone A (**10**).[38]

The isolated compounds were evaluated for cytotoxicity against the human colorectal adenocarcinoma cell line HT-29, and their IC_{50} values are shown in Table 5.

Table 5. Cytotoxicity of the Isolated Compounds from the Roots of *P. falcatus*

compound	IC_{50} against HT-29 (μM)[a]
1	0.6 ± 0.4
2	1.1 ± 0.5
3	0.3 ± 0.1
4	>10
5	>10
6	0.9 ± 0.3
7	5.1 ± 0.8
8	0.5 ± 0.1
9	>10
10	>10
Taxol	0.00082 ± 0.0003

[a]The values represent the average ± standard deviation from a triplicate.

From the strong antiproliferative data displayed by all the nagilactones it may be inferred that (1) the γ-lactone moiety together with the unsaturated δ-lactone unit of nagilactones is responsible for the strong antiproliferative activity, (2) introduction of a lipophilic ester group at C-16/17 slightly lowers the activity, while hydroxylation of the isopropyl side chain of nagilactones decreases the activity roughly 2- to 3-fold, and (3) the presence of a C-1/C-2 epoxide moiety marginally decreases the activity. These observations were in good agreement with a previous report on the structure–activity relationship of natural and synthetic nagilactones against the *Yoshida sarcoma* cell line.[39]

In summary, the present study led to isolation of six bioactive and four inactive compounds through bioassay-guided fractionation and isolation, three of which are new. The traditional use of the plant may be justified by its high content of cytotoxic nagilactones. The isolation of compounds **1** and **2** from the roots of *P. falcatus* adds a number to the rare type C nagilactones, which are the most cytotoxic among the nagilactones.[39,40]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a PerkinElmer model 343 polarimeter. UV spectra were recorded on a Hitachi U2910 UV spectrophotometer. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer. ^1H and ^{13}C, HSQC, HMBC, NOESY, and COSY NMR spectra were recorded at room temperature on a Bruker AVIII400 instrument. HRESIMS were measured on a Q-TOF mass spectrometer in the positive-ion mode. Sephadex LH-20 (GE, Uppsala, Sweden) and silica gel (Silica-FlashP60, 230–400 mesh) were used for open-column chromatography. Analytical TLC was performed using precoated silica gel 60 F_{254} plates (200 μm, Merck). HPLC was performed with a Hitachi Primaide HPLC equipped with a Primaide 1430 diode array detector, a Primaide 1210 autosampler, and a Primaide 1110 pump with a degasser and a semipreparative C_{18} column (Dynamax C_{18} HPLC column, 10 × 250 mm).

Plant Material. Roots of *P. falctus* were collected from the Berga forest, Addis Alem (55 km west of Addis Ababa), Oromia Region, Ethiopia, in March 2014. Identification of the plant material was done by Mr. Melaku Wondafrash, Senior Botanist at The National Herbarium, Addis Ababa University, Addis Ababa, Ethiopia. Voucher specimens (EMA004) were deposited at The National Herbarium, Addis Ababa University, Ethiopia.

Extraction and Isolation. The air-dried roots of *P. falcatus* (270 g) were extracted with 90% MeOH (3 × 0.6 L) by maceration at room temperature for nine consecutive days with solvent renewal every 72 h. Removal of the solvents using a Rotavapor and lyophylizer yielded a brownish, gummy solid (13 g), which showed cytotoxicity against HT-29 with an ED_{50} value of 10 μg/mL. Liquid–liquid partition with hexanes, EtOAc, and H_2O yielded the corresponding hexanes (1.21 g, ED_{50} = 11 μg/mL), EtOAc (2.15 g, ED_{50} = 3 μg/mL), and H_2O (9.44 g, ED_{50} >20 μg/mL) fractions. The most active EtOAc fraction (ED_{50} = 3 μg/mL) was partitioned with dichloromethane (DCM, 5 × 500 mL) and H_2O (400 mL) to afford 1.01 g of DCM (ED_{50} = 2 μg/mL) and 1.06 g (ED_{50} > 20 μg/mL) of H_2O fractions. Using DCM/MeOH (1:1) as eluent, the cytotoxic DCM fraction was subjected to Sephadex LH-20 CC (4.5 × 62 cm; flow rate = 2.5 mL/min) to afford 111 fractions. Fractions with similar TLC profiles were combined to yield seven pooled fractions (F1–F7), with F3 being the most active (ED_{50} = 0.7 μg/mL). The fraction F3 was subjected to silica gel CC (3.2 cm × 39 cm; flow rate = 3.5 mL/min) to afford 14 pooled fractions (F3-1 through F3-14). Cytotoxicity evaluation of these fractions revealed that F3-4 and F3-5 were the most active, with equal ED_{50} values of 0.4 μg/mL. These fractions were subjected to semipreparative RP-18 HPLC using a gradient mixture of H_2O/MeOH from 50:50 to 0:100 (flow rate: 2 mL/min). Fraction F3-4 (46.3 mg) yielded compounds **1** (1.3 mg; t_R = 16.12 min), **3** (11.2 mg; t_R = 15.30 min), and **4** (11.7 mg; t_R = 27.00 min), while F3-5 (83 mg) yielded compounds **3** (3 mg; t_R = 15.30 min) and **4** (4.13 mg; t_R = 27.00 min) and an impure fraction (41.47 mg; t_R = 10.07 min), which was resubjected to semipreparative RP-18 HPLC (gradient mixture of H_2O/MeOH from 90:10 to 0:100) to yield compounds **6** (11.47 mg; t_R = 30.35 min) and **8** (12.89 mg; t_R = 31.43 min). Preparative RP-18 HPLC using a gradient of H_2O/MeOH (from 20:80 to 0:100) of F3-1 (30.2 mg) yielded compound **5** (3.7 mg; t_R = 22.53 min), whereas RP-18 HPLC purification of F3-9 yielded compounds **7** (0.9 mg; t_R = 8.19 min), **2** (1.58 mg; t_R = 10.81 min), and **10** (6.08 mg; t_R = 25.49 min) using a gradient of H_2O/MeOH (from 60:40 to 0:100). Compound **9** (1 mg) was obtained as a precipitate from one of the inactive fractions after column chromatography.

*16-Hydroxynagilactone F (**1**):* fine colorless needles; $[\alpha]_D^{20}$ −78 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3388 (OH), 1762 (γ-lactone), 1701 (δ-lactone), 1597 (conjugated C=C) cm^{-1}; UV (MeOH) λ_{max} (log ε) 259 (3.7); ^1H and ^{13}C NMR data, see Table 3 and Table 4; HRESIMS $[M + Na]^+$ *m*/*z* 355.1500 (calcd for $C_{19}H_{24}O_5Na^+$, 355.1516).

*2β,16-Dihydroxynagilactone F (**2**):* fine colorless needles; $[\alpha]_D^{20}$ −75 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3390 (OH), 1764 (γ-lactone), 1698 (δ-lactone), 1600 (conjugated C=C) cm^{-1}; UV (MeOH) λ_{max} (log ε) 261 (4.06); ^1H and ^{13}C NMR data, see Table 3 and Table 4; HRESIMS $[M + Na]^+$ *m*/*z* 371.1460 (calcd for $C_{19}H_{24}O_6Na^+$, 371.1465).

*7β-Hydroxymacrophyllic acid (**4**):* white, amorphous solid; $[\alpha]_D^{20}$ +66.9 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3533, 3359–2400 (broad), 2958, 2932, 2872, 1694, 1467, 1453, 1259, 1175 cm^{-1}; UV λ_{max} (MeOH) (log ε) 217 (4.45), 251 (3.96), 291 (3.67) nm; ^1H and ^{13}C NMR data, see Table 3 and Table 4; HRESIMS $[M + Na]^+$ *m*/*z* 685.3727 (calcd for $C_{40}H_{54}O_8Na^+$, 685.3711).

X-ray Crystallographic Data of Compounds 3 and 8. Crystals of compounds **3** and **8** were obtained from MeOH upon slow evaporation. In each case a colorless crystal was mounted on the goniometer of an Agilent Nova diffractometer operating with Cu Kα radiation (λ = 1.541 84 Å). The data collection routine, unit cell refinement, and data processing were carried out with the program CrysAlisPro.[41] The structure was solved using SHELXT-2014[42] and refined using SHELXL-2014 via OLEX.[42] The final refinement model involved anisotropic displacement parameters for non-hydrogen

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atoms. A riding model was used for the aromatic and alkyl hydrogens. Hydrogen atom positions and isotropic displacement parameters were refined independently for the hydroxy and water hydrogens that are potentially involved in hydrogen bonding. The absolute configuration was established from anomalous dispersion effects for **3** [Flack x = −0.07(13);[43] Hooft P2(true) = 1.000, P3(true) = 1.000, P3(rac-twin) = 0.000; P3(false) = 0.000, y = −0.00(11)][44−46] and for **8** [Flack x = −0.13(8);[43] Hooft P2(true) = 1.000, P3(true) = 1.000, P3(rac-twin) = 0.000; P3(false) = 0.000, y = −0.09(7)].[44−46] Olex2 was used for molecular graphics generation.[46] The details of the structural analyses are described in the Supporting Information along with the CIF files, which have also been deposited in the Cambridge Crystallographic Data Centre (CCDC Nos. 1038573 and 1038572 for compounds **3** and **8**, respectively).

Crystal data of ***3****:* $C_{19}H_{24}O_5$, M = 332.38, orthorhombic crystal system, crystal size 0.44 × 0.13 × 0.23 mm^3, space group $P2_12_12_1$, a = 7.8220(2) Å, α = 90°, b = 12.4501(4), β = 90°, c = 17.1803(6) Å, γ = 90°, V = 1673.11(9) Å^3, reflections collected 10 392, parameters 225.

Crystal data of ***8****:* $C_{20}H_{24}O_7$, M = 376.39, orthorhombic crystal system, crystal size 0.14 × 0.19 × 0.28 mm^3, space group $P2_12_12_1$, a = 7.77930(10) Å, α = 90°, b = 11.77260(10) Å, β = 90°, c = 19.7352(3) Å, γ = 90°, V = 1807.40(4) Å^3, reflections collected 11 384, parameters 252.

Bioassay. The cytotoxicity of the isolated compounds was screened against HT-29 cells by a previously reported procedure.[47]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary UV, IR, 1D and 2D NMR, and HRESIMS spectra for compounds **1**, **2**, **3**, **4**, and **8**, the X-ray structure of compound **6**, as well as the CIF data of compounds **3** and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1 614 292 4733. Fax: +1 614 292 2435. E-mail: rakotondraibe.1@osu.edu (L. H. Rakotondraibe).

Notes

The authors declare no competing financial interest.

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