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Bioactive Formylated Flavonoids from *Eugenia rigida*: Isolation, Synthesis, and X-ray Crystallography

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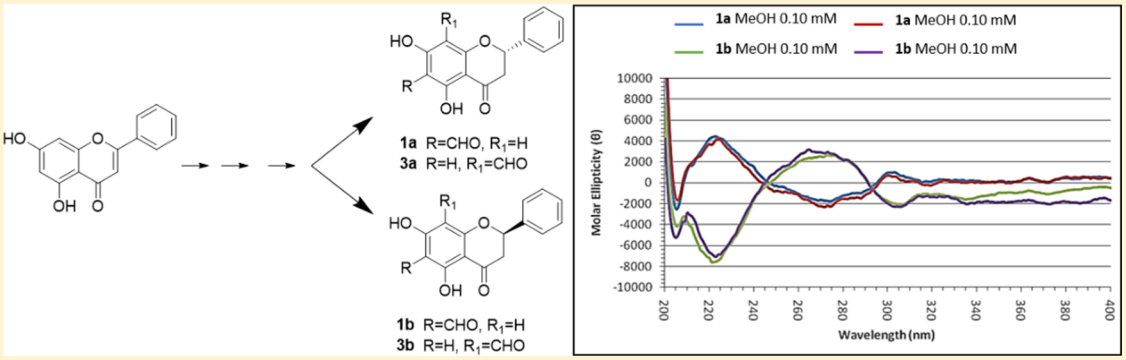
Supporting Information



ABSTRACT: Two new flavonoids, *rac*-6-formyl-5,7-dihydroxyflavanone (**1**) and 2′,6′-dihydroxy-4′-methoxy-3′-methylchalcone (**2**), together with five known derivatives, *rac*-8-formyl-5,7-dihydroxyflavanone (**3**), 4′,6′-dihydroxy-2′-methoxy-3′-methyldihydrochalcone (**4**), *rac*-7-hydroxy-5-methoxy-6-methylflavanone (**5**), 3′-formyl-2′,4′,6′-trihydroxy-5′-methyldihydrochalcone (**6**), and 3′-formyl-2′,4′,6′-trihydroxydihydrochalcone (**7**), were isolated from the leaves of *Eugenia rigida*. The individual (*S*)- and (*R*)-enantiomers of **1** and **3**, together with the corresponding formylated flavones **8** (6-formyl-5,7-dihydroxyflavone) and **9** (8-formyl-5,7-dihydroxyflavone), as well as 2′,4′,6′-trihydroxychalcone (**10**), 3′-formyl-2′,4′,6′-trihydroxychalcone (**11**), and the corresponding 3′-formyl-2′,4′,6′-trihydroxydihydrochalcone (**7**) and 2′,4′,6′-trihydroxydihydrochalcone (**12**), were synthesized. The structures of the isolated and synthetic compounds were established via NMR, HRESIMS, and electronic circular dichroism data. In addition, the structures of **3**, **5**, and **8** were confirmed by single-crystal X-ray diffraction crystallography. The isolated and synthetic flavonoids were evaluated for their antimicrobial and cytotoxic activities against a panel of microorganisms and solid tumor cell lines.

Plants belonging to the genus *Eugenia* (Myrtaceae) produce a wide array of bioactive secondary metabolites, including flavonoids,[1,2] triterpenoids,[3] phenolic compounds,[1] chromones,[4] and sterols.[5] Flavonoids are widely consumed as common ingredients of the human diet, and many of them are reputed phytonutrients for cancer chemoprevention, as well as to protect cells from oxidative damage and to reduce the risks of diseases associated with oxidative stress. On the basis of epidemiological evidence, dietary flavonoids present in fruits, vegetables, and spices may lower the prevalence of colon, lung, prostate, breast, and pancreatic cancers.[6−8] Various types of flavonoids have been isolated from plants in the genus *Eugenia*, including *E. uniflora*[9] and *E. edulis*.[10] Cytotoxic and antitumor activities have also been reported for *E. jambolana*,[11] *E. floccosa*, and *E. singampattiana*.[12] In addition, *E. operculata*, also known as *Cleistocalyx operculaus*,[13] contains formylated flavonoids[14−16] with proven cytotoxic[14] and antioxidant activities.[14,15] Various formylated flavanones, flavones, and dihydrochalcones (DHCs) have been reported from *Psidium acutangulum*,[17−19] *Friesodielsia discoclor*,[20] *Nicotiana tabacum*,[21] and *Ziziphus jujuba*[22] and were demonstrated to display cytotoxic,[20,21] antioxidant, and antifungal activities.[17−19]

The isolation of three resveratrol analogues from *Eugenia rigida* DC. (Myrtaceae), a shrub or small tree used in folk medicine for leukemia in Argentina, their cytotoxic and antioxidant activity, and modulation of cancer-related signaling pathways were previously reported.[23] In the course of a continuing search for bioactive secondary metabolites from *E. rigida*, the isolation and characterization of the new flavonoids,

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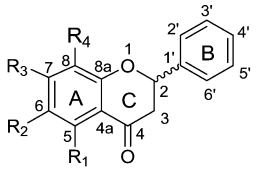
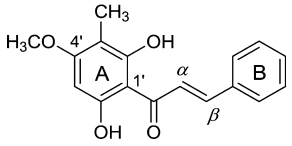
rac-6-formyl-5,7-dihydroxyflavanone (**1**) and 2′,6′-dihydroxy-4′-methoxy-3′-methylchalcone (**2**), together with five known compounds, namely, *rac*-8-formyl-5,7-dihydroxyflavanone (**3**), 4′,6′-dihydroxy-2′-methoxy-3′-methyldihydrochalcone (**4**), *rac*-7-hydroxy-5-methoxy-6-methylflavanone (**5**), 3′-formyl-2′,4′,6′-trihydroxy-5′-methyldihydrochalcone (**6**), and 3′-formyl-2′,4′,6′-trihydroxydihydrochalcone (**7**), are reported herein. In addition, the (*S*)- and (*R*)-enantiomers of **1** and **3**, 6-formyl-5,7-dihydroxyflavone (**8**), 8-formyl-5,7-dihydroxyflavone (**9**), 2′,4′,6′-trihydroxychalcone (**10**), 3′-formyl-2′,4′,6′-trihydroxychalcone (**11**), DHC (**7**), and 2′,4′,6′-trihydroxydihydrochalcone (**12**) were synthesized, and the structures of **3**, **5**, and **8** were confirmed by single-crystal X-ray diffraction crystallography. The antimicrobial and cytotoxic activities of the isolated and synthetic compounds were evaluated.



	R_1	R_2	R_3	R_4
1	OH	CHO	OH	H
3	OH	H	OH	CHO
5	OCH_3	CH_3	OH	H



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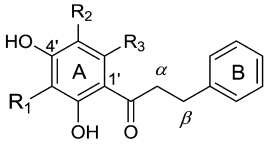

	R_1	R_2	R_3
4	H	CH_3	OCH_3
6	CH_3	CHO	OH
7	H	CHO	OH

RESULTS AND DISCUSSION

A bioassay-guided fractionation procedure of the EtOH extract of *E. rigida*, using centrifugal preparative thin-layer chromatography (CPTLC), afforded compounds **1**−**7**. The ^{1}H and ^{13}C NMR spectroscopic data of compounds **3** and **5** suggested the presence of a flavanone skeleton, while the spectra of **4**, **6**, and **7** suggested their dihydrochalcone backbones. The structures of **3**,[20] **4**,[24] **5**,[25] **6**,[17] and **7**[18,26] were established by comparison of their observed and reported data.

Compound **1**, obtained as colorless needles, showed a protonated molecular ion peak at *m*/*z* 285.0637 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0763) in its HRESIMS data, suggesting the molecular formula $C_{16}H_{12}O_5$. The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) of **1** were similar to those of the known 8-formyl-5,7-dihydroxyflavanone (**3**) except for the presence of the −CHO group at C-6 (δ_H 10.2, 1H, s; δ_C 192.0, s), instead of C-8 in **3** (δ_H 10.1, 1H, s; δ_C 191.2, s), suggesting that compounds **1** and **3** are regioisomeric. The ^{1}H NMR spectrum of **1** showed two geminally coupled signals at δ_H 2.91 (1H, dd, J = 17.2 and 3.2 Hz; H-3eq) and 3.13 (1H, dd, J = 17.2 and 12.4 Hz; H-3ax), which were coupled vicinally to a proton resonating at δ_H 5.50 (1H, dd, J = 3.2 and 12.4 Hz; H-2). The HMQC spectrum revealed that these proton signals correlated to a methylene [δ_C 42.9 (C-3)] and an oxygenated methine [δ_C 79.6 (C-2)] carbon, respectively, reminiscent of the ABX spin system of the protons of the heterocyclic ring of flavanones. The ^{1}H NMR spectrum of **1** also showed signals at δ_H 7.48−7.52 (5H, m) for the protons of an unsubstituted B-ring and at δ_H 10.2 (1H, s), 6.02 (1H, s), 13.25 (1H, s), and 12.77 (1H, s), which were assigned to a −CHO group, an aromatic proton, and two hydrogen-bonded hydroxy groups, respectively. The HMBC experiment showed correlations of the OH-5 and -7 protons at δ_H 13.25 and 12.77 and the formyl proton at δ_H 10.2 to an sp^2-hybridized carbon at δ_C 101.6 (C-6), confirming the location of the formyl group at C-6. The HMBC spectrum showed ^2J correlations between the aromatic proton at δ_H 6.02 and C-7 and C-8a (δ_C 171.1 and 167.7, respectively), supporting its placement at C-8. From the aforementioned data and the observed optical inactivity, the structure of compound **1** was defined as *rac*-6-formyl-5,7-dihydroxyflavanone.

The HRESIMS data of **2**, a yellow powder, showed a protonated molecular ion peak at *m*/*z* 285.1133 $[M + H]^+$ (calcd for $C_{17}H_{17}O_4$, 285.1127], attributed to a molecular formula of $C_{17}H_{16}O_4$. The ^{1}H and ^{13}C NMR data (Table 2) showed two doublets for an olefinic AB spin system at δ_H 7.85 and 7.98 (J_{AB} = 15.6 Hz) and a carbonyl carbon at δ_C 193.1, indicating the presence of an α,β-unsaturated carbonyl group, a characteristic spectroscopic feature of the chalcone class of natural products.[27] Two sets of multiplets integrating for five protons at δ_H 7.42 (3H) and 7.66 (2H) and a one-proton singlet at δ_H 6.22 (H-5′) were assigned to an unsubstituted B-ring and a tetrasubstituted A-ring, respectively. ^{1}H NMR signals for a methoxy group at δ_H 3.70 (s, OCH_3-4′), a hydrogen-bonded phenolic group at δ_H 13.32 (s, OH-6′), and a methyl group at δ_H 2.14 (s, CH_3-3′) confirmed the presence of three of the A-ring substituents. The ^{13}C NMR data of **2** were consistent with those of a chalcone with tetra- and unsubstituted A- and B-rings, respectively. The two olefinic carbons resonated at δ_C 126.4 (C-α) and 143.3 (C-β) and the carbonyl carbon at δ 193.1. In addition, signals for a methoxy [δ_C 62.2 (OCH_3-4′)], a methyl [δ_C 8.0 (CH_3-3′)], and three oxygenated sp^2-hybridized carbons at δ_C 161.3 (C-2′), 161.4 (C-4′), and 164.1 (C-6′) attributed to the tetrasubstituted A-ring were evident. Initially, the HMBC experiment with a delay time of 50 ms was performed to establish the ^2J and ^3J correlations. Cross-peaks between H-5′ (δ 6.22) and C-1′, C-3′, C-4′, and C-6′ and between the methyl protons (δ_H 2.14) and C-2′, C-3′, and C-4′ confirmed the location of the methyl group at C-3′. Subsequently, this experiment was repeated with a delay time of 200 ms to obtain ^4J correlations, which showed two additional cross-peaks between H-5′ (δ_H 6.22) and the methoxy (δ_C 62.2) and carbonyl (δ_C 193.1) carbons. These experiments established unequivocally that the methoxy group is attached to C-4′. Thus, the structure of compound **2** was defined as 2′,6′-dihydroxy-4′-methoxy-3′-methylchalcone.

The C-6 and C-8 formylated flavanones **1** and **3** were isolated as racemic mixtures. In order to obtain sufficient quantities for the bioactivity studies, the (*S*)- and (*R*)-enantiomers of **1** and **3** together with the related flavone analogues were synthesized (Scheme 1). The (*S*)- and (*R*)-

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Table 1. ^{1}H and ^{13}C NMR Data for 6-Formyl-5,7-dihydroxyflavanone (1) and 8-Formyl-5,7-dihydroxyflavanone (3)[a]

	1			3		
no.	δ_C, type	δ_H, (J in Hz)	HMBC[b]	δ_C, type	δ_H (J in Hz)	HMBC[b]
2	79.6, CH	5.50, dd (3.2, 12.4)		80.5, CH	5.60, dd (3.2, 13.2)	
3eq	42.9, CH_2	2.91, dd (17.2, 3.2)	4	42.6, CH_2	2.94, dd (17.2, 3.2)	4
3ax		3.13, dd (17.2, 12.4)	2, 4		3.18, dd (17.2, 13.2)	2, 4, 1′
4	195.9, C			195.0, C		
5	168.7, C			169.3, C		
6	101.6, C			97.4, CH	6.05, s	8
7	171.1, C			170.7, C		
8	96.2, CH	6.02, s	6, 7, 4a, 8a	101.7, C		
8a	167.7, C			166.5, C		
4a	105.3, C			104.4, C		
1′	137.5, C			137.0, C		
2′	126.2, CH	[c]	2, 4, 6′	126.0, CH	[d]	2, 4′
3′	129.1, CH	[c]	1′, 5′	129.0, CH	[d]	4′
4′	129.4, CH	[c]	2′, 6′	129.4, CH	[d]	
5′	129.1, C	[c]	1′, 3′	129.0, CH	[d]	4′
6′	126.2, CH	[c]	2, 2′, 4′	126.0, CH	[d]	2, 4′
OH-5		13.25, s	5, 6, 4a		12.75, s	5, 6, 10
CHO-6	192.0, CH	10.20, s	7			
OH-7		12.77, s	6, 7, 8		12.57, s	6, 7, 8
CHO-8				191.2, CH	10.10, s	7, 8

[a]Spectra run at 400 MHz (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$. [b]HMBC correlations are from proton(s) stated to the indicated carbon. [c]7.48−7.52 (5H, m). [d]7.45−7.50 (5H, m).

Table 2. ^{1}H and ^{13}C NMR Data for 2′,6′-Dihydroxy-4′-methoxy-3′-methylchalcone (2) and 4′,6′-Dihydroxy-2′-methoxy-3′-methyldihydrochalcone (4)[a]

	2			4[b]		
no.	δ_C, type	δ_H (J in Hz)	HMBC[c]	δ_C, type	δ_H (J in Hz)	HMBC[c]
1	135.2, C			141.4, C		
2	128.4, CH	[d]	4, 6, β	128.4, CH	[f]	1, 6, β
3	128.9, CH	[e]	1, 2	128.4, CH	[f]	1, 5
4	130.0, CH	[e]	2, 6	125.9, CH	[f]	2, 6
5	128.9, CH	[e]	1, 6	128.4, CH	[f]	1, 3
6	128.4, CH	[d]	2, 4, β	128.4, CH	[f]	2, β
α	126.3, CH	7.98, d (15.6)	1, β, CO	44.2, CH_2	3.40, t (7.6)	1, β, CO
β	143.2, CH	7.85, d (15.6)	1, 2, 6, α, CO	30.6, CH_2	3.03, t (7.6)	1, 2, 6, α, CO
1′	109.6, C			109.3, C		
2′	161.3, C			161.6, C		
3′	110.1, C			109.5, C		
4′	161.4, C			160.9, C		
5′	99.7, CH	6.22, s	1′, 3′, 4′, 6′	99.7, CH	6.19, s	3′, 4′, 6′
6′	164.1, C			163.5, C		
OCH_3-2′				61.6, OCH_3	3.71, s	6′
OCH_3-4′	62.2, CH_3	3.70, s	4′			
CH_3-3′	8.0, CH_3	2.14, s	2′, 3′, 4′	8.4, CH_3	2.10, s	2′, 3′, 4′
OH-6′		13.32, s	1′, 2′, 3′		13.11, s	1′, 5′, 6′
C═O	193.1			204.8		

[a]Spectra run at 400 MHz (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$. [b]Data of this compound are presented to compare with **2**. [c]HMBC correlations are from proton(s) stated to the indicated carbon. [d]7.66 (2H, m). [e]7.42 (3H, m). [f]7.22−7.32 (5H, m).

enantiomers of 5,7-dihydroxyflavanone (pinocembrin), **13a** and **13b**, were synthesized according to a reported procedure.[28] Commercially available 5,7-dihydroxyflavone (chrysin) **14** was hydrogenated with 10% Pd−C to give a racemic mixture of pinocembrin **13**. Benzylation of the 7-hydroxy group and subsequent imination with the chiral auxiliary (L)-(−)-α-methylbenzylamine using $TiCl_4$ as catalyst afforded a separable mixture of diastereoisomers. The mixture was chromatographed to give imines **15a** and **15b**. Hydrolysis of imines **15a** and **15b** afforded, in turn, the (*S*)- and (*R*)-flavanones **16a** and **16b**. Debenzylation of compounds **16a** and **16b** via hydrogenolysis afforded the pinocembrin enantiomers (*S*)-**13a** and (*R*)-**13b**, respectively.

Although there is a wide range of approaches available for the introduction of an aromatic formyl group,[29] the formylation of pinocembrin proceeded slowly due to deactivation of the A-ring via the electron-withdrawing effect of the C-4 carbonyl group. Standard procedures such as Reimer−Tiemann, Duff, Gatter-

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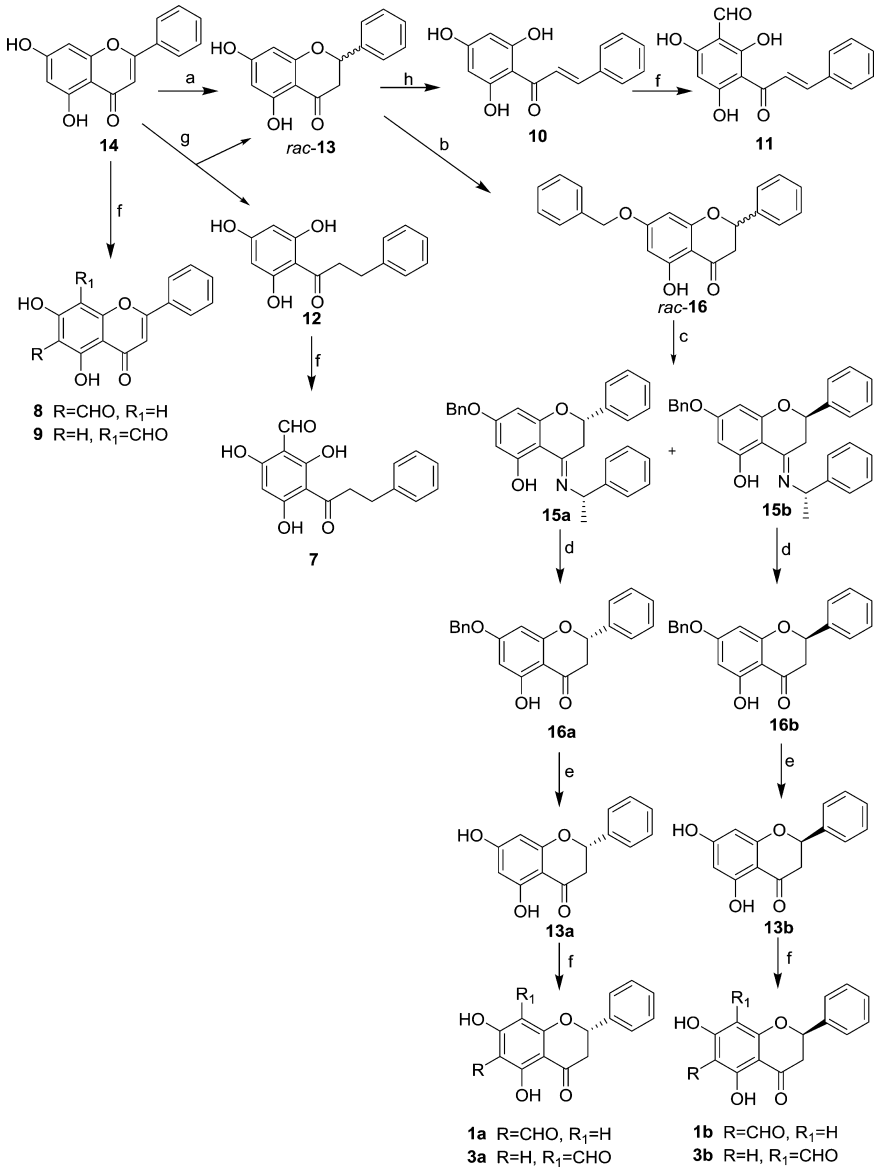
Scheme 1. Synthesis of the Formylated Flavonoids[a]



[a]Reagents and conditions: (a) Pd−C (10%), H_2, EtOH, 60 °C, 2 h, 3 atm; (b) BnCl, K_2CO_3, KI, acetone, reflux, 2 h; (c) L-(−)-α-methylbenzylamine, Et_3N, $TiCl_4$, benzene, N_2, 48 h; (d) HCl (1 N), EtOH, reflux, 1.5 h; (e) Pd−C (10%), H_2, DMF, HCl (1 N), 4 h; (f) dichloromethyl methyl ether, $TiCl_4$, CH_2Cl_2, −70 to 0 °C, 2.5−4 h; (g) Pd−C (10%), H_2, EtOH, 60 °C, 2 h, 30 atm; (h) 4 M KOH, EtOH, 0 °C.

mann, Vilsmeier, and triethyl orthoformate/$AlCl_3$ formylations afforded low yields. The best results were obtained by utilizing dichloromethyl methyl ether (Cl_2CHOMe) as the C_1 source and $TiCl_4$ as the Lewis-acid catalyst.[30] Formylation of **13a** under these conditions afforded a mixture of regioisomers, which was separated by preparative HPLC to afford (2*S*)-6-formyl-5,7-dihydroxyflavanone **1a** (25%) and its regioisomer (2*S*)-8-formyl-5,7-dihydroxyflavanone **3a** (9%). Using the same procedures (2*R*)-6-formyl-5,7-dihydroxyflavanone **1b** and (2*R*)-8-formyl-5,7-dihydroxyflavanone **3b** (9%) were prepared from **13b**. The structures of the enantiomers **1a**, **1b**, **3a**, and **3b** were elucidated by comparison of their physical and spectroscopic data with those of the isolated compounds **1** and **3**. The absolute configurations were defined via their electronic circular dichroism (ECD) spectroscopic data (vide infra).[31]

Formylation of chrysin **14** using the $Cl_2CHOMe/TiCl_4$ procedure afforded the 6- and 8-formylchrysins **8** and **9**. Their structures were confirmed from their NMR spectroscopic data (Table 3). Hydrogenation of chrysin (**14**) at high pressure yielded a mixture of 2′,4′,6′-trihydroxydihydrochalcone (**12**) and the racemic flavanone **13**. Formylation of **12** afforded 3′-formyl-2′,4′,6′-trihydroxydihydrochalcone (**7**). Treatment of **13** with KOH yielded 2′,4′,6′-trihydroxychalcone (**10**), for which the formylation afforded 3′-formyl-2′,4′,6′-trihydroxychalcone (**11**).

The absolute configurations of the enantiomeric flavanone pair **1a** and **1b** were assessed via their chiroptical data. The ECD curves of compound **1a** showed sequential positive and negative Cotton effects near 310 and 280 nm for the n → π* and π → π* electronic transitions, respectively. These Cotton effects are reminiscent of flavanones exhibiting *P*-helicity of the conformationally flexible heterocycle with a C-2 equatorial B-ring and, hence, (2*S*) absolute configuration.[32] The mirror image related ECD spectrum of **1b** accordingly confirmed its

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Table 3. ^{1}H and ^{13}C NMR Data for 6-Formyl-5,7-dihydroxyflavone (8) and 8-Formyl-5,7-dihydroxyflavone (9)[a]

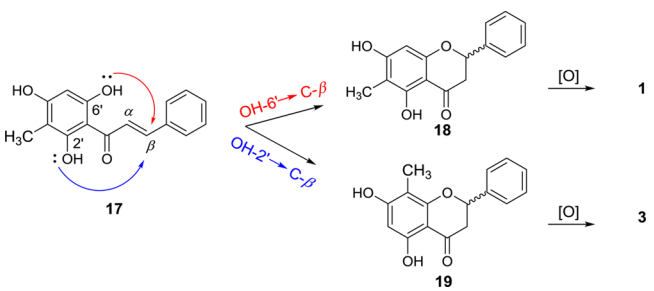
	8			9		
no.	δ_C, type	δ_H (J in Hz)	HMBC[b]	δ_C, type	δ_H (J in Hz)	HMBC[b]
2	164.7, C			163.9, C		
3	105.7, CH	6.69, s	2, 4, 10, 1′	107.1, CH	6.79, s	2, 10, 1′
4	182.5, C			181.5, C		
5	167.6, C			168.4, C		
6	106.6, C			100.0, CH	6.32, s	7, 10
7	167.8, C			169.4, C		
8	94.7, CH	6.44, s	6, 7, 9, 10	103.2, C		
8a	161.6, C			171.8, C		
4a	103.9, C			104.5, C		
1′	130.5, C			130.4, C		
2′	126.3, CH	7.88, m	2, 4′, 6′	126.2, CH	7.84, m	2, 4′, 6′
3′	129.2, CH	7.57, m	1′, 5′	129.4, CH	7.58, m	5′
4′	130.5, CH	7.57, m	2′, 6′	132.5, CH	7.58, m	2′, 6′
5′	129.2, C	7.57, m	1′, 3′	129.4, CH	7.58, m	3′
6′	126.3, CH	7.88, m	2, 2′, 4′	126.2, CH	7.84, m	2, 2′, 4′
OH-5		14.24, s	5, 6, 10		13.60, s	5, 6, 10
CHO-6	192.4, CH	10.36, s	6, 7			
OH-7		12.45, s	6, 7, 8		12.66, s	6, 7, 8
CHO-8				189.3, CH	10.32, s	7

[a]Spectra run at 400 MHz (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$. [b]HMBC correlations are from proton(s) stated to the indicated carbon.

(2*R*) absolute configuration. The (2*S*) enantiomer **1a** displayed a high-amplitude positive Cotton effect near 230 nm corresponding to the $^{1}L_a$ electronic transitions of the aromatic chromophores. Conversely, the negative Cotton effect displayed by compound **1b** in the same wavelength region confirmed its (2*R*) absolute configuration. These assignments were supported by the observed levo- and dextrorotatory characteristics of **1a** and **1b**, respectively.[33] A notable feature of the ECD spectra and UV data of these formylflavanones is that the Cotton effects and the UV absorption maxima are all blue-shifted compared to flavanones devoid of the formyl substituent. Such shifts presumably reflect the contributions of the benzaldehyde component of the molecular chromophore also comprising the acetophenone and B-ring aromatic structural moieties.

It should be noted that a 2′,4′,6′-trihydroxy-3-methylchalcone of type **17** presumably served as the biosynthetic precursor to the regioisomeric formylflavanones **1** and **3**. The cyclization step would proceed via an intramolecular 1,4-Michael addition reaction involving both HO-2′ and HO-6′, similar to the scattering of the A-ring substituents during the Wessely–Moser rearrangement of appropriately substituted flavanones. Subsequent oxidation of the benzylic methyl groups would afford the formylated flavanones **1** and **3** (Scheme 2).

Scheme 2. Putative Biosynthetic Route toward the Formation of 1 and 3



The structure of *rac*-**3** as determined by X-ray diffraction analysis is shown in Figure 1. Both hydroxy groups form

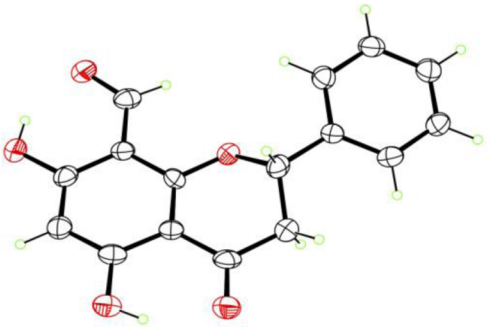


Figure 1. X-ray structure of **3** with 50% ellipsoids.

intramolecular hydrogen bonds, HO-5 to the flavanone carbonyl (O···O 2.571(4) Å) and HO-7 to the formyl carbonyl (O···O 2.603(4) Å). The formyl group lies essentially in the plane of the flavanone aromatic ring, with a C–C–C–O torsion angle of 2.4(9)°, and the stereogenic C-2 atom lies 0.61 Å out of plane. The structure of racemic **5** is shown in Figure 2. A disorder exists in which C-2 has the opposite configuration with 26% occupancy, which also changes the position of the phenyl substituent. The HO-5 group forms an intermolecular hydrogen bond to the flavanone carbonyl, O···O 2.715(2) Å. The structure of flavone **8** is shown in Figure 3. As in **3**, the formyl substituent lies in the plane of the aromatic A-ring [C–C–C–O torsion angle −0.7(4)°]. Both OH groups again form intramolecular hydrogen bonds, with O···O distances of 2.540(3) and 2.595(2) Å, the longer one being to the formyl group.

In vitro antimicrobial and antifungal activities were evaluated for the isolated and synthetic compounds (Table 4). Antimicrobial activities were evaluated against the bacteria *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium*

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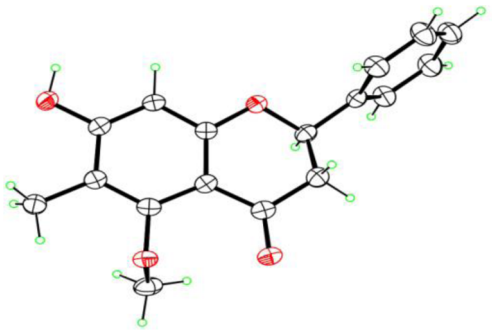

Figure 2. X-ray structure of **5** with 50% ellipsoids. Only the main component of the disorder is shown.

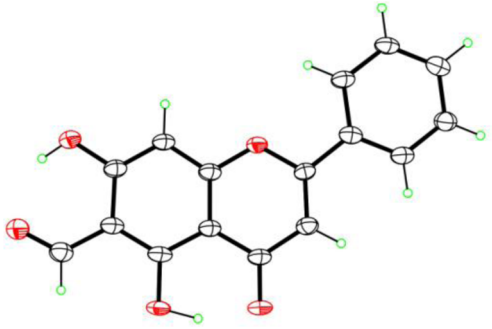

Figure 3. X-ray structure of **8** with 50% ellipsoids.

intracellulare and the fungi *Candida albicans*, *C. glabrata*, *C. krusei*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. Antistaphylococcal activities against *S. aureus* and MRSA were observed for dihydrochalcone **7** (IC_{50} of 17.4 and 36.6 μM, respectively). On the other hand, antifungal activity was observed for chalcone **11** against *C. albicans*, *C. glabrata*, *C. krusei*, and *C. neoformans* (IC_{50} values of 68.9, 10.0, 34.1, and 24.5 μM, respectively), while dihydrochalcone **7** was active only against *C. glabrata* and *C. neoformans*, with IC_{50} values of 11.3 and 10.5 μM, respectively. However, compounds **1a**, **1b**, **3a**, **3b**, and **13** were active against *C. glabrata* and *C. neoformans*. Finally, all of the isolated and synthetic compounds were tested for cytotoxicity against selected human cancer cell lines, namely, skin melanoma (SK-MEL), epidermal carcinoma (KB), breast carcinoma (BT-549), ovarian carcinoma (SK-OV-3), and cervical carcinoma (HeLa), as well as two noncancer cell lines [African green monkey kidney (VERO) and pig kidney epithelial ($LLC\text{-}PK_1$) cells]. Doxorubicin was used as the positive control. All compounds tested were inactive (IC_{50} > 10 μM) for all of the cell lines used.

This appears to be the first report of the formylated flavanone **1** and the methylchalcone **2** from a natural source, as well as the first report of the synthesis of the (2*S*)- and (2*R*)-enantiomers of **1** and **3**, formylated flavone **8**, and chalcone **11**, and the X-ray crystal structures of compounds **3**, **5**, and **8**. Among the synthetic compounds, **8**, **9**, and **11** were not reported from natural sources previously. The isolation of flavonoids **1**–**7**, together with the known cytotoxic and antioxidant resveratrol analogues (*Z*)- and (*E*)-3,4,3′,5′-tetramethoxystilbene and (*E*)-3,5,4′-trimethoxystilbene,[23] illustrates the potential of *E. rigida* as a source of potential antimicrobial compounds.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Mettler FP 51 apparatus. Optical rotations were recorded at ambient temperature using a Rudolph Research Analytical Autopol IV automatic polarimeter. UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV/vis spectrometer, and ECD data were collected using an Olis Cary-17 spectrophotometer (1 cm path length cell). IR spectra were obtained using a Bruker Tensor 27 instrument. NMR spectra were acquired on a Varian Mercury 400 MHz NMR spectrometer at 400 (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$, using the residual solvent as an internal standard. Multiplicity determinations (DEPT) and 2D NMR spectra (HMQC, HMBC, NOESY) were obtained using standard Bruker pulse programs. HRESIMS data were acquired by direct injection using a Bruker Bioapex-FTMS with electrospray ionization (ESI). TLC was carried out on precoated silica gel 60 F254 (EMD Chemicals Inc., Darmstadt, Germany) using toluene–EtOAc (9:1) and *n*-hexane–EtOAc (7.5:2.5) as solvents. Centrifugal preparative TLC (CPTLC, using a Chromatotron, Harrison Research Inc. model 8924, tagged with a fraction collector) was carried out on 6 mm custom-made reversed-phase (RP) C_{18} silica gel,[34] 1, 2, and 4 mm silica gel P254 (Analtech) rotors, using H_2O in MeOH, EtOAc in *n*-hexane, and $CHCl_3$ as eluents. SPE cartridges C_{18} (Supelco Inc., Bellefonte, PA, USA) were used in the fractionation experiments. Final purifications were performed on preparative HPLC silica gel columns (silica gel-100 A 250 × 15.00, 5 μM) from Phenomenex Luna (Torrance, CA, USA) using an HPLC Delta Prep 4000 (Waters Corporation, Milford, MA,

Table 4. Antimicrobial Activity of Isolated and Synthetic Compounds

	IC_{50}/MIC/MFC/MBC[a]						
compound	*C. albicans*	*C. glabrata*	*C. krusei*	*A. fumigatus*	*C. neoformans*	*S. aureus*	MRSA
E. rigida EtOH ext	>50	11.27	20.98	>50	27.7	>50	>50
1a	–/–/–	6.7/35.2/70.4	33.2/–/–	–/–/–	63.2/70.4/–	–/–/–	–/–/–
1b	–/–/–	12.6/70.4/70.4	–/–/–	–/–/–	61.5/–/–	–/–/–	–/–/–
3a	–/–/–	7.4/35.2/–	29.9/–/–	–/–/–	44.4/70.4/70.4	–/–/–	–/–/–
3b	–/–/–	7.2/35.2/70.4	41.2/–/–	–/–/–	19.3/–/–	–/–/–	–/–/–
7	–/–/–	11.3/69.9/–	–/–/–	–/–/–	10.5/17.4/17.4	17.4/–/–	36.6/–/–
8	–/–/–	10.8/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–
13	–/–/–	42.9/–/–	65.5/–/–	–/–/–	28.8/78.1/–	–/–/–	–/–/–
11	68.9/–/–	10.0/35.2/70.4	34.1/–/–	–/–/–	24.5/70.4/70.4	–/–/–	–/–/–
Amb	0.2/0.3/0.7	0.2/0.7/0.7	0.6/1.3/1.4	1.4/2.7/5.4	0.2/0.3/0.3	ND/ND/ND	ND/ND/ND
Cipro	ND/ND/ND	ND/ND/ND	ND/ND/ND	ND/ND/ND	ND/ND/ND	0.3/1.5/1.5	0.2/0.8/3

[a]IC_{50} is the concentration in μM that affords 50% growth relative to controls; MIC (minimum inhibitory concentration) is the lowest concentration that allows no growth; MFC (minimum fungicidal concentration) or MBC (minimum bactericidal concentration) is the lowest concentration at kills the test organism; ND = no data; –/–/– not active at the highest test concentration of 20 μg/mL; Amb = amphotericin B; Cipro = ciprofloxacin.

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USA) equipped with a dual-wavelength detector model 2487 adjusted at 210 and 254 nm. Samples were dried using a Savant Speed Vac Plus SC210A concentrator. The compounds were visualized by spraying the TLC plates with 1% vanillin−H_2SO_4 spray reagent. The reference standard doxorubicin (purity ≥98.0% pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant Material. The leaves of *E. rigida* were collected in Guanica, Puerto Rico, in March 2006. The sample was identified by Mr. F. Axelrod, and a voucher specimen (MOBOT 3008783) was deposited in the Herbarium of the Missouri Botanical Garden, St. Louis, MO, USA.

Extraction and Isolation. The dried and pulverized leaves of *E. rigida* (107 g) were macerated in *n*-hexane and subsequently extracted with CH_2Cl_2 (3 × 600 mL, sonicated for 2 h and then left overnight). The combined *n*-hexane and CH_2Cl_2 extracts were filtered separately and dried (yields 2.50 and 3.87 g, respectively). A portion of the *n*-hexane extract (2 g) was subjected to CPTLC, using a 6 mm custom-made RP-C_{18} silica gel disk.[34] The sample was dissolved in CH_3CN, and applied on a presaturated (30% MeOH−H_2O) disk on a Chromatotron apparatus, and, after sample application, the disk was kept in a desiccator. The semidried disk was mounted on the Chromatotron and was eluted with a step gradient of H_2O−MeOH (from 30% to 0% H_2O). Fractions of 60 mL each were collected and monitored by TLC (silica gel, solvent: toluene−EtOAc, 9:1). Fractions 1−11, eluted with 30% H_2O−MeOH, were combined, evaporated to dryness (560 mg), and subjected to another CPTLC run, using a 4 mm silica gel disk (Analtech). Elution was carried out with *n*-hexane−EtOAc mixtures with increasing polarity to afford compounds **1** (3 mg), **3** (3 mg), and **4** (1 mg). A portion of the CH_2Cl_2 extract (2 g) was subjected to fractionation on an SPE cartridge filled with 50 g of C_{18}-silica gel and eluted with a step gradient of H_2O−MeOH (from 100% to 0% H_2O in MeOH). Fractions of 100 mL each were collected and monitored by TLC (silica gel, solvent: *n*-hexane−EtOAc, 7.5:2.5). Fraction 5 (20 mg), eluted with 70% MeOH−H_2O, was chromatographed over preparative HPLC using the gradient mobile phase *n*-hexane−EtOAc of increasing polarities to yield **5** (2 mg). Fractions 11 and 12, eluted with 75% MeOH−H_2O, were combined and dried (60 mg), then subjected to CPTLC, using a 1 mm silica gel disk, eluted with $CHCl_3$, to yield 15 subfractions. Subfraction 14 was subjected to purification by preparative HPLC to afford compound **2** (3 mg). Fractions 19−22, eluted with 75% MeOH−H_2O, were combined, dried (80 mg), and then subjected to CPTLC, using a 2 mm silica gel disk, eluted with $CHCl_3$, to yield 16 subfractions. Subfraction 8 afforded compound **6** (1 mg), while subfraction 13 was subjected to further purification by preparative HPLC to afford compound **7** (5 mg).

*rac-6-Formyl-5,7-dihydroxyflavanone (**1**):* colorless needles ($CHCl_3$); mp 160−162 °C; $[\alpha]_D$ 0 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.3), 267 (4.5), 331 (3.6) nm; IR ν_{max} (KBr) 2920, 1661 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS (+ve) *m*/*z* 285.0637 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0685).

*2′,6′-Dihydroxy-4′-methoxy-3′-methylchalcone (**2**):* yellow, amorphous powder ($CHCl_3$); UV (MeOH) λ_{max} (log ε) 325 (4.1) nm; ^1H and ^{13}C NMR spectral data, see Table 2; HRESIMS (+ve) *m*/*z* 285.0667 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0695).

*4′,6′-Dihydroxy-2′-methoxy-3′-methyldihydrochalcone (**4**):* yellow, microcrystalline powder ($CHCl_3$); mp 205−207 °C; IR ν_{max} (KBr) 2921, 2360, 1661 cm^{-1}; ^1H and ^{13}C NMR data, Table 2.

Synthesis of Compounds 1a and 3a. To a solution of **13a**[28] (150 mg, 0.59 mmol) and $TiCl_4$ (160 μL, 1.46 mmol) in dry CH_2Cl_2 (10 mL) at −78 °C was added dropwise dichloromethyl methyl ether (58 μL, 0.65 mmol) with stirring under N_2. After 2 h, the reaction mixture was warmed to room temperature over 1 h. Aqueous H_2SO_4 acid (5%, 20 mL) was added to the reaction mixture, and the organic phase was separated. The aqueous phase was extracted with ether (3 × 20 mL), the combined organic phase was dried over $MgSO_4$, and the solvent was removed in vacuo. The residue was chromatographed by preparative HPLC on silica gel using a mobile phase of *n*-hexane (A) and EtOAc (B) in a gradient mode: A:B 100−75:25 for 35 min, 75:25−0:100 for 20 min, to give compounds **1a** (43 mg, 25%) and **3a** (15 mg, 9%).

*(2S)-6-Formyl-5,7-dihydroxyflavanone (**1a**):* colorless needles ($CHCl_3$); mp 160−162 °C; $[\alpha]_D$ −31 (*c* 0.5, MeOH); ECD (MeOH) λ (θ) 223 (4.44 × 10^3), 274 (−1.76 × 10^3), 303 (1.02 × 10^3) nm; ^1H and ^{13}C NMR spectra in agreement with compound **1**; HRESIMS *m*/*z* 285.083 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0685).

*(2S)-8-Formyl-5,7-dihydroxyflavanone (**3a**):* colorless needles ($CHCl_3$); mp 160−162 °C; $[\alpha]_D$ −31 (*c* 0.5, MeOH); ^1H and ^{13}C NMR spectra in agreement with the isolated compound **3**; HRESIMS, *m*/*z* 285.0628 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0685).

Synthesis of Compounds 1b and 3b. Application of the same formylation and separation procedures using **13b**[28] afforded **1b** (43 mg, 25%) and **3b** (15 mg, 9%).

*(2R)-6-Formyl-5,7-dihydroxyflavanone (**1b**):* colorless needles ($CHCl_3$); mp 160−162 °C; $[\alpha]_D$ +31 (*c* 0.5, MeOH); ECD (MeOH) λ (θ) 223 (−7.07 × 10^3), 274 (2.83 × 10^3), 303 (−2.26 × 10^3) nm; ^1H and ^{13}C NMR spectra in agreement with the isolated compound **1**; HRESIMS *m*/*z* 285.0637 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0685).

*(2R)-8-Formyl 5,7-dihydroxyflavanone (**3b**):* colorless needles ($CHCl_3$); mp 160−162 °C; $[\alpha]_D$ +31 (*c* 0.5, MeOH); ECD (MeOH) λ (θ) 223 (−8.27 × 10^3), 276 (2.27 × 10^4), 332 (−2.36 × 10^3) nm; ^1H and ^{13}C NMR data in agreement with compound **3**; HRESIMS *m*/*z* 285.0637 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0685).

Synthesis of Compounds 8 and 9. Application of the same formylation and separation procedures using **14** afforded **8** (40 mg, 24%) and **9** (15 mg, 9%).

*6-Formyl-5,7-dihydroxyflavone (**8**):* yellow needles; mp 144−146 °C; ^1H and ^{13}C NMR data (Table 3); HRESIMS *m*/*z* 283.0566 $[M + H]^+$ (calcd for $C_{16}H_{11}O_5$, 283.0528).

*8-Formyl-5,7-dihydroxyflavone (**9**):* yellow needles; mp 144−146 °C; ^1H and ^{13}C NMR data (Table 3); HRESIMS *m*/*z* 283.0534 $[M + H]^+$ (calcd for $C_{16}H_{11}O_5$, 283.0528).

Synthesis of 2′,4′,6′-Trihydroxychalcone (10). A solution of compound **13** (256 mg, 1 mmol) in EtOH (10 mL) was added dropwise to a stirred solution of 4 M KOH (10 mL) at 0 °C under Ar, and the reaction mixture was kept at room temperature for 30 min. The mixture was poured into ice water (10 mL), acidified with 1 M HCl to pH 3−4, and extracted with EtOAc (3 × 10 mL). The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness The residue was purified by silica gel column chromatography (*n*-hexane−EtOAc, 80:20) to give compound **10** (141 mg, 55%) as a yellow, microcrystalline powder; mp 142−143 °C; HRESIMS *m*/*z* 257.0778 $[M + H]^+$ (calcd for $C_{15}H_{13}O_4$, 257.0769).

Synthesis of 3′-Formyl-2′,4′,6′-trihydroxychalcone (11). Formylation of **10** by the aforementioned procedure yielded **11**, which was purified by silica gel chromatography (*n*-hexane−EtOAc, 90:10) to afford a yellow powder (30 mg, 34%): ^1H NMR (400 MHz, methanol-d_4) δ_H 10.08 (1H, s, CHO), 8.14 (1H, d, H-α, *J* = 16.0 Hz), 7.80 (1H, d, H-β, *J* = 16.0 Hz), 7.66 (2H, m, H-2, H-6), 7.42 (3H, m, H-3, H-4, H-5), 5.86 (1H, s, H-5′); HRESIMS *m*/*z* 285.0646 $[M + H]^+$ (calcd for $C_{16}H_{13}O_5$, 285.0685).

Synthesis of 2′,4′,6′-Trihydroxydihydrochalcone (12). A mixture of compound **14** (508 mg, 2 mmol) and 10% Pd−C (1 g) in EtOH (150 mL) was hydrogenated at 30 atm, for 2 h, at 60 °C. After filtration, the solvent was evaporated under reduced pressure, and the residue was chromatographed over silica gel (*n*-hexane−EtOAc, 80:20) to afford compounds **9** (230 mg, 45%) and **12** (180 mg, 35%) as yellow, amorphous powders: ^1H NMR (400 MHz, methanol-d_4) δ_H 7.23−7.12 (5H, m, Ar−H), 5.82 (2H, d, H-3′ and H-5′, *J* = 1.6 Hz), 3.30 (2H, t, H-α, *J* = 7.5 Hz), 2.92 (2H, t, H-β, *J* = 7.5 Hz); HRESIMS *m*/*z* 259.0899 $[M + H]^+$ (calcd for $C_{15}H_{15}O_4$, 259.0926).

Synthesis of 3′-Formyl-2′,4′,6′-trihydroxydihydrochalcone (7). Formylation of **12** by the procedure described above followed by purification by silica gel chromatography (*n*-hexane−EtOAc, 90:10) yielded **7** (58 mg, 35%) as a yellow powder: ^1H NMR (400 MHz, methanol-d_4) δ_H 10.09 (1H, s, CHO), 7.63 (5H, m, Ar−H), 5.85 (1H,

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s, H-5′), 3.47 (2H, t, H-α, J = 7.6 Hz), 3.04 (2H, t, H-β, J = 7.6 Hz); HRESIMS m/z 287.0700 $[M + H]^+$ (calcd for $C_{16}H_{15}O_5$, 287.0841).

X-ray Crystallography of Compounds 3, 5, and 8. The crystal structures of **3**, **5**, and **8** were determined, using data collected at T = 90 K with Cu Kα radiation on a Bruker APEX-II DUO CCD diffractometer, equipped with a microfocus source and an Oxford Cryostream cooler. The structures were solved using SHELXS-97[35] and refined anisotropically by full-matrix least-squares on F^2 using SHELXL-97.[35] H atoms were visible in difference maps, but were placed in idealized positions for the refinement, except for those of OH groups, which were refined for **5** and **8**. In **5**, a disorder exists in which both enantiomers are superimposed, causing the stereogenic C atom to occupy two positions separated by 1.07 Å, with populations 0.74:0.26. The phenyl group attached to this C atom also takes on two orientations. Compound **5** was also a nonmerohedral twin. Crystal data: **3**, $C_{16}H_{12}O_5$, M_r = 284.26, orthorhombic space group $Pna2_1$, a = 12.3875(10) Å, b = 23.583(2) Å, c = 4.2613(4) Å, V = 1244.88(19) Å^3, Z = 4, D_x = 1.517 Mg m^{-3}, θ_{max} = 62.6°, R = 0.049 for 1140 data and 193 refined parameters; CCDC 1452453. **5**: $C_{17}H_{16}O_4$, M_r = 284.30, monoclinic space group $P2_1/c$, a = 13.1877(7) Å, b = 14.1222(6) Å, c = 7.9446(4) Å, β = 106.644(3)°, V = 1417.61(12) Å^3, Z = 4, D_x = 1.332 Mg m^{-3}, θ_{max} = 67.0°, R = 0.050 for 2425 data and 224 refined parameters; CCDC 1452454. **8**: $C_{16}H_{10}O_5$, M_r = 282.24, monoclinic space group $P2_1/n$, a = 12.0917(11) Å, b = 5.1819(4) Å, c = 19.6180(13) Å, β = 94.901(6)°, V = 1224.73(17) Å^3, Z = 4, D_x = 1.531 Mg m^{-3}, θ_{max} = 66.6°, R = 0.059 for 2094 data and 196 refined parameters; CCDC 1452455.

Antimicrobial Assays. All organisms were obtained from the American Type Culture Collection (Manassas, VA, USA) and included the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) method.[36−38] *M. intracellulare* was tested using a modified Franzblau method.[39] Samples were serially diluted in 20% DMSO−saline and transferred in duplicate to 96-well flat-bottomed microplates. Microbial inocula were prepared by correcting the OD_{630} of microbe suspensions in incubation broth to give final target inocula. Drug controls [ciprofloxacin (ICN Biomedicals, Aurora, OH, USA) for bacteria and amphotericin B (ICN Biomedicals) for fungi] were included in each assay. All organisms were read at either 530 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments, Winooski, VT, USA) or 544ex/590em (*M. intracellulare, A. fumigatus*) using the Polarstar Galaxy plate reader (BMG Lab Technologies, Ortenburg, Germany) prior to and after incubation. Minimum fungicidal or bactericidal concentrations were determined by removing 5 μL from each clear well, followed by transferring to agar, and incubating. The MFC/MBC is defined as the lowest test concentration that kills the organism (allows no growth on agar).

Cytotoxic Assay. The in vitro cytotoxic activity was determined against five human cancer cell lines (SK-MEL, KB, BT-549, SKOV-3, and HeLa) and two noncancerous kidney cell lines (VERO and LLC-PK1). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The assay was performed in 96-well tissue culture-treated microplates. Cells were seeded at a density of 25 000 cells/well and incubated for 24 h. Samples at different concentrations were added, and cells were again incubated for 48 h. At the end of incubation, the cell viability was determined using Neutral Red dye according to a modification of the procedure of Borenfreund et al.[40] IC_{50} values were determined from dose−response curves of percent decrease in cell viability against test concentrations. Doxorubicin was used as a positive control and exhibited IC_{50} values of 1.5, 3.8, 3.6, 2.6, 4.0, >10, and 17 for the SK-MEL, KB, BT-549, SKOV-3, HeLa, VERO, and LLC-PK1 cell lines, respectively. DMSO was used as the negative (vehicle) control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00474.

NMR spectra of the isolated and synthetic compounds, ECD spectra of the enantiomeric compounds **1a**/**1b**, and X-ray crystallographic data of compounds **3**, **5**, and **8** (PDF)

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Notes

The authors declare no competing financial interest.

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