Bioactive Constituents from *Iryanthera megistophylla*

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Activity-guided fractionation of the 95% ethanol extract from the stem bark of *Iryanthera megistophylla* led to the isolation of two new compounds, named megislignan [2,3-dimethyl-4-(4-methoxyphenyl)-6-hydroxynaphthalene] (**1**) and megislactone [(2*R*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(hexacos-17-enyl)butanolide] (**2**), along with seven known compounds, grandinolide (**3**), iryantherin K (**4**), iryantherin L (**5**), cinchonain I b (**6**), cinchonain I a (**7**), procyanidin B-2 (**8**), and cinchonain IIa (**9**). The structures of the new compounds were elucidated by spectral data interpretation. Isolates were evaluated for their antibacterial, antifungal, antiviral, and antiacetylcholinesterase activities.

Iryanthera megistophylla A. C. Sm. is in the Myristicaceae, a family rich in flavonoids and lignans. Previous chemical studies carried out on Iryanthera species have led to the isolation of lignans,[1] flavonolignans,[1−3] γ-lactone derivatives,[4] and tocotrienols.[5] There have been reports of Amazonian natives using some *Iryanthera* species for medicinal healing. The crushed leaves of some species have been used to treat seriously infected wounds, and the latex of the bark has been mixed with water for the treatment of gastric infections.[6] The species *I. megistophylla* is used in the treatment of cutaneous leishmaniasis by Afro-Colombians on the Pacific coast of Colombia. Its crude methanol extract exhibited strong activity against the herpes simplex virus.[7] The bioactive chemical constituents of this species, however, have not been reported. This report describes the bioactivity-guided isolation of phytochemicals from the bark of *I. megistophylla* and the structural determination of two new compounds, named megislignan (2,3-dimethyl-4-(4-methoxyphenyl)-6-hydroxynaphthalene) (**1**) and megislactone [(2*R*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(hexacos-17-enyl)butanolide] (**2**), along with seven known compounds, grandinolide [(2*S*,3*S*,4*S*)-3-hydroxy-4-methyl-2-(17-pheneyl-*n*-heptadecyl)butanolide] (**3**),[4] iryantherins K (**4**) and L (**5**),[3] cinchonains Ib (**6**) and Ia (**7**),[7] procyanidin B-2 (**8**),[8] and cinchonain IIa (**9**),[8] as well as the evaluation of antibacterial, antifungal, antiviral, and antiacetylcholinesterase activities of compounds **1**−**9** (Chart 1).

Results and Discussion

Extraction of *I. megistophylla* with 95% ethanol followed by solvent partition resulted in the localization of the active components in the ethyl acetate fraction. Repeated column chromatography followed by crystallization and preparative TLC afforded the isolation of nine compounds (**1**−**9**) including two new compounds (**1** and **2**). The structures of the known compounds were identified by comparison of their ^{1}H and ^{13}C NMR data with those reported in the literature. The elucidation of the structures of the new compounds **1** and **2** is presented herein.

Compound **1** was obtained as an amorphous powder, mp 189−190 °C, $[\alpha]^{20}_D$ −18° (*c* 1.0, $CHCl_3$). The HREIMS of **1** gave a molecular ion at *m*/*z* 278.13092 corresponding to a molecular formula of $C_{19}H_{18}O_2$. The IR spectrum of **1** indicated the presence of aromatic rings (1602, 1508, 1464 cm^{-1}). The ^{1}H NMR spectrum (in CD_3COCD_3) of **1** showed eight olefinic and aromatic proton resonances, of which the signals at δ 6.58 (1H, d, J = 2.2 Hz), 7.00 (1H, dd, J = 8.8, 2.2 Hz), and 7.65 (1H, dd, J = 8.8 Hz) suggested the existence of an 1,3,4-trisubstituted phenyl ring. The other four aromatic protons at δ 7.08 (2H, dd, J = 8.8, 2.4 Hz) and 7.09 (2H, dd, J = 8.8, 2.4 Hz) implied that the structure of **1** contains another aromatic ring with 1,4-disubstituted groups. An olefinic proton signal occurred as a singlet at δ 7.56, suggesting that no proton was affixed to the adjacent carbon. The two methyl groups appearing at δ 2.40 (3H, s) and 2.08 (3H, s) indicated that they are linked directly to the double bond. The ^{13}C NMR spectrum of **1** and DEPT experiments confirmed that the molecule contained 19 carbons including three methyls, eight methines, and eight quaternary carbons, of which the signals at δ 134.6 (C-4a), 108.7 (C-5), 155.4 (C-6), 117.8 (C-7), 129.4 (C-8), and 128.0 (C-8a) were attributed to the 1,3,4-trisubstituted phenyl ring by combining the analysis of their ^{1}H−^{1}H COSY and HMQC spectral data. The signals at δ 133.6, 132.0 (×2), 114.6 (×2), and 159.6 confirmed the presence of a 1,4-disubstituted phenyl ring. The HMBC NMR spectrum (see Figure 1) showed long-range carbon to hydrogen connectivities from C-1 to H-9 and H-8; from C-8 to H-1; from C-9 to H-1; from C-4 to H-10, H-5, H-2′, and H-6′; and from C-4′ to the protons of the methoxy group, leading to the structural determination of compound **1** as 2,3-dimethyl-4-(4-methoxyphenyl)-6-hydroxynaphthalene.

Compound **2** was isolated as a white amorphous powder. A molecular formula of $C_{31}H_{58}O_3$ was established for **2** from its HREIMS. Analysis of the IR spectrum of **2** suggested the presence of a five-membered lactone ring (1759 cm^{-1}). The ^{1}H NMR spectrum of **2** (Table 1) exhibited signals for two methyl groups at δ 0.86 (t, J = 6.5 Hz) and 1.39 (d, J = 6.5 Hz), a methine group at δ 2.52 (m), and two oxymethine groups at δ 4.18 (dd, J = 3.9, 4.6 Hz) and 4.61 (qd, J = 6.5, 4.6 Hz). The connectivities of these groups were derived from an analysis of the ^{1}H−^{1}H COSY, HMQC,

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Chart 1

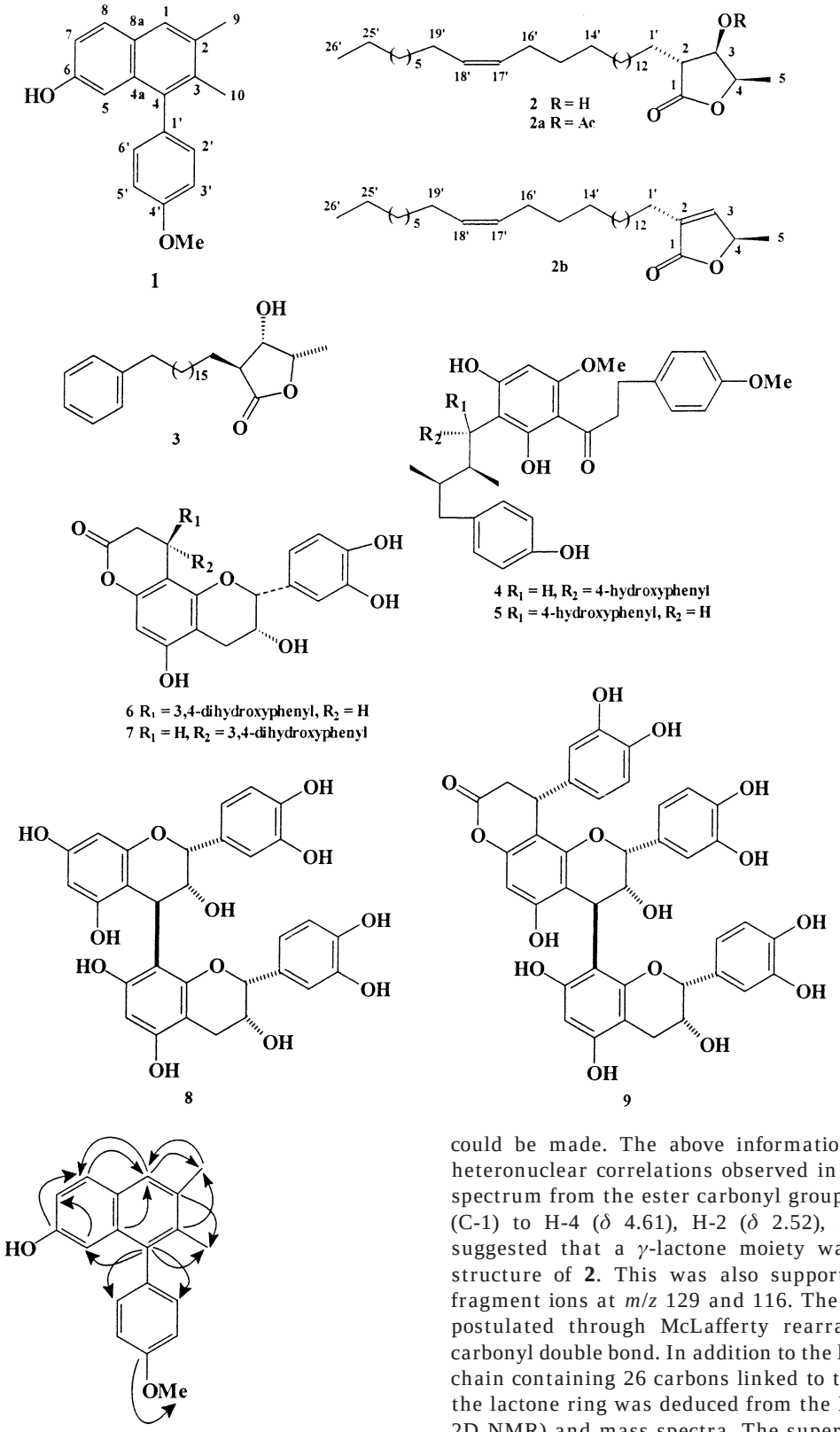

Figure 1. Selected HMBC correlations for **1** (arrows denote HMBC correlations from C to H).

and HMBC NMR spectra. In the $^1H-^1H$ COSY spectrum, the double doublet at δ 4.18 (H-3) showed cross-peaks with the protons at δ 2.52 (H-2) and 4.61 (H-4). The latter also coupled with a triplet at δ 1.39 (H-5). These correlations revealed the connectivities of C-2 to C-5. From the ^{13}C NMR and HMQC spectra, assignments of these four carbons could be made. The above information and long-range heteronuclear correlations observed in the HMBC NMR spectrum from the ester carbonyl group signal at δ 177.6 (C-1) to H-4 (δ 4.61), H-2 (δ 2.52), and H-3 (δ 4.18) suggested that a γ-lactone moiety was present in the structure of **2**. This was also supported by the EIMS fragment ions at *m/z* 129 and 116. The latter ion may be postulated through McLafferty rearrangements of the carbonyl double bond. In addition to the lactone ring, a side chain containing 26 carbons linked to the C-2 position in the lactone ring was deduced from the NMR (^1H, ^{13}C and 2D NMR) and mass spectra. The superimposable signals at δ 5.33 (2H, dt, J = 9.4, 4.6 Hz) in the ^1H NMR spectrum and δ 129.9 (2C) in the ^{13}C NMR spectrum indicated that a double bond was present in the side chain. The coupling constant between the two olefinic protons was 9.4 Hz, suggesting its *cis* geometry. The EIMS fragment ions at *m/z* 99, 153, and 154, generated from the allylic cleavage (*m/z* 99, 153) and McLafferty rearrangements (*m/z* 154) of the double bond, respectively, together with the series of

Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1** and **2**[a]

	1			**2**	
position	δ_H (mult., *J*/Hz)	δ_C	position	δ_H (mult., *J*/Hz)	δ_C
1	7.56 (s)	127.7 (d)	1		177.6 (s)
2		132.7 (s)	2	2.52 (m)	49.2 (d)
3		134.2 (s)	3	4.18 (dd, 3.9,4.6)	74.1 (d)
4		137.3 (s)	4	4.61 (qd, 6.5, 4.6)	78.2 (d)
4a		134.6 (s)	5	1.39 (d, 6.5)	14.1 (q)
5	6.58 (d, 2.2)	108.7 (d)	1′a	1.70 (m)	28.4 (t)
6		155.4 (s)	1′b	1.45 (m)	
7	7.00 (dd, 8.8, 2.2)	117.8 (d)	2′	1.51 (m)	29.0 (t)
8	7.65 (d, 8.8)	129.4 (d)	3′−15′	1.20−1.38 (m)	29.3−31.9 (t)
8a		128.0 (s)	16′	2.00 (m)	27.2 (t)
9	2.40 (s)	20.9 (q)	17′	5.33 (dt, 9.4, 4.6)	129.9 (d)
10	2.08 (s)	17.7 (q)	18′	5.33 (dt, 9.4, 4.6)	129.9 (d)
1′		133.6 (s)	19′	2.00 (m)	27.2 (t)
2′, 6′	7.08 (dd, 8.8, 2.4)	132.0 (d)	20′−25′	1.20−1.38 (m)	29.3−31.9 (t)
3′, 5′	7.09 (dd, 8.8, 2.4)	114.6 (d)	26′	0.86 (t, 6.5)	13.9 (q)
4′		159.6 (s)			
OCH3	3.88 (s)	55.5 (q)			

[a] Spectra were recorded in CD_3COCD_3 for **1** and $CDCl_3$ for **2**; chemical shifts are reported as δ values (ppm) from TMS at 400 MHz for ^{1}H and 100 MHz for ^{13}C; signal multiplicity and coupling constants (Hz) are shown in parentheses.

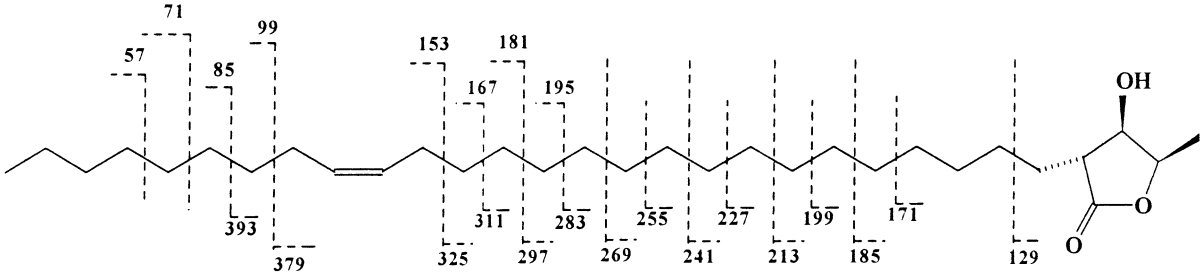

Figure 2. EIMS fragmentation (*m*/*z* values) for compound **2**.

peaks at *m*/*z* 325, 311, 297, 283, 269, 255, 241, 227, 213, 199, 185, 171, and 129 (see Figure 2) indicated that this *cis* double bond is situated between C-17′ and C-18′ of the side chain.

The relative stereochemistry of these five-membered ring lactones has been determined by using ^{13}C NMR analyses.[10,11] The ^{13}C chemical shift for the methyl group is δ 13.8 ± 0.2 when it is *cis* to the hydroxyl group on C-3 and δ 18.0 ± 0.2 when it is *trans*. The methylene group at C-1′ appears at δ 27.8 ± 0.6 when it is *trans* to the hydroxyl group on C-3 and at δ 23.2 ± 0.2 when they are *cis* to each other.[10,11] The lanthanide-induced shifts and NOE experiments are in agreement with these results.[4,10,12] Compound **2**, with the ^{13}C NMR chemical shifts at δ 14.1 and 28.4 for the methyl group (C-5) and the methylene group (C-1′), respectively, was deduced to have the *cis* (between methyl and hydroxyl) and *trans* (between hydroxyl and C-1′) configurations in the lactone ring. The relative stereochemistry of H-2, H-3, and H-4 in **2** was further confirmed using NOE difference experiments. The enhancements between H-3 and H-4, and H-3 and H-26 (CH_2), and absence of enhancement between H-2 and H-3 demonstrated that H-3, H-4, and C-26 are *cis* oriented and H-2 and H-3 are *trans* oriented. The absolute configurations of lactones such as **2** could be deduced by comparison of the $[\alpha]_D$ signals with those of the compounds of known sterechemistry.[4,10,13−15] Compound **2** showed negative values (laevorotatory), thus its absolute configuration was postulated as (2*R*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(hexacos-17-enyl)butanolide, which was further confirmed by the following evidence. Compound **2** gave an endocyclic α,β-unsaturated-γ-lactone **2b** by successive acetylation (Ac_2O, pyridine) and elimination of the acetic acid (Al_2O_3, C_5H_{12}). Compound **2b** was laevorotatory ($[\alpha]_D^{25}$ −25°). Its absolute configuration at C-3 was determined as *R* by comparison with the laevoratatory compound of known absolute stereochemistry: (4*R*)-2-dodecyl-4-methylbut-2-enolide) ($[\alpha]_D^{22}$ −29.8°).[14,15]

Growth inhibitory effects against *Staphylococcus aureus* and *Candida albicans* were evaluated for compounds **1**−**9** using a disk diffusion assay. Determination of minimal inhibitory concentrations (MIC) against *S. aureus* gave values of 50 μg/mL for compound **4** and 100 μg/mL for compound **8**. Determination of MIC against *C. albicans* resulted in a value of 150 μg/mL for compounds **6**−**9**. The remaining compounds did not exhibit activity against *C. albicans* or *S. aureus* at the upper concentration tested in the broth dilution assay (300 and 200 μg/mL, respectively) (Table 2).

Compounds **1**−**9** were also tested for their anti-HSV-1 (herpes simplex virus type 1) activity. Compounds **6** and **7** exhibited potent activity against HSV-1 at a minimal concentration of 20 μg/mL (Table 2). No cytotoxicity was found for these two compounds (up to 400 μg/mL). Compounds **6** and **7** are thus at least in part responsible for the antiviral activity detected in the crude extract of *I. megistophylla*.[7]

On the basis of the high level of anti-potato virus X (PVX) activity shown previously by similar compounds,[16] compounds **1**−**9** were tested for their anti-potato virus X activity by means of a local lesion assay. Table 2 shows the results. Of the compounds tested, **4** and **6**−**9** showed very high levels of inhibition against PVX. Compounds **6**−**9** showed higher levels of inhibition than ribavirin, an antiviral compound that has activity against a wide range of plant viruses, including PVX.[17] Due to its similar

Table 2. Activities of Compounds **1**−**9** against Herpes Simplex Virus (HSV-1), a Fungus (*C. albicans*), a Bacterium (*S. aureus*), and Potato Virus X (PVX)

compound	HSV-1 (MIC, μg/mL)	*C. albicans* (MIC, μg/mL)	*S. aureus* (MIC, μg/mL)	PVX % inhibition (% error)[d]
1	*a*	*b*	*c*	−36 (57))
2	*a*	*b*	*c*	20 (35)
3	*a*	*b*	*c*	30 (43)
4	*a*	*b*	50	89 (15)
5	*a*	*b*	*c*	67 (39)
6	20	150	*c*	100 (0)
7	20	150	*c*	100 (0)
8	*a*	150	100	98 (5)
9	*a*	150	*c*	100 (0)
quercetin				100 (0)
ribavirin				94 (7)

[a] No detectable activity at 320 μg/mL. [b] No detectable activity at 300 μg/mL. [c] No detectable activity at 200 μg/mL. [d] Error was calculated as standard deviation between results on each leaf. The high error of compounds **1**−**3** and **5** is due to their nonspecific effect toward PVX infection.

structure and previously reported antiviral activity,[16] quercetin was used as an additional positive control for this study.

Compounds **1** and **4** also exhibited antiacetylcholinesterase activity, with **4** showing 77.4 ± 6.1% inhibition and **1** showing 67.6 ± 3.4% inhibition of acetylcholinesterase, respectively, when tested at a concentration of 0.8 mg/mL. The activity of compounds **1** and **4** is considered moderate when compared to the 100.0 ± 0.0% inhibition displayed by galanthamine, an approved acetylcholinesterase inhibitor. When tested at 0.16 mg/mL the level of acetylcholinesterase inhibition was significantly reduced, with compounds **1** and **4** showing 5.1 ± 2.2 and 10.6 ± 2.5% inhibition, respectively, while galanthamine exhibited 99.7 ± 0.1% inhibition. Acetylcholinesterase inhibitors have been shown to increase mental efficiency and are prescribed as a therapy for Alzheimer's disease.[18] (Due to limitations in the amounts of isolated compounds, only compounds **1** and **4** were tested.)

Aside from the activities reported here, compound **6** has also been shown to be hepatoprotective[19,20] and to display cytotoxity against leukemia cells.[21] Compound **7** has demonstrated 5-lipoxygenase inhibition assay, which indicates its potential activity as an antiinflammatory agent.[22] Compound **8** exhibited antileishmanial activity in vitro,[23] which may explain the traditional use of *I. megistophylla* in the treatment of leishmaniasis.

Experimental Section

General Experimental Procedures. Melting points were determined with a Gallenkamp melting point apparatus and are uncorrected. Optical rotations were obtained on a JASCO P-1010 polarimeter. IR spectra were obtained using KBr disks on a BOMEM MB-100 spectrophotometer. UV spectra were obtained on a Unicam 8700 series UV/vis spectrometer. NMR spectra were run on a Bruker Avance-400 MHz spectrometer. EIMS and HREIMS were recorded on a Kratos MS 50 mass spectrometer. Si gel (Merck, 200−400 mesh) was used for column chromatography. Thin-layer chromatography analysis was carried out on Si gel GF254 plates (Merck). Preparative TLC was performed using Si gel 60 GF254 (Merck, 250 μm thickness).

Plant Material. The bark of *I. megistophylla* A.C. Sm. (AL-29) was collected in Bajo Calima, Department of Valle del Cauca, Colombia, in July 1999 and identified by Dr. R. Callejas, Antioquia University Herbarium (Medellin, Colombia). A voucher specimen (López 29) is kept in the herbarium of the Sinchi Institute (Santa Fe de Bogotá, Colombia).

Extraction and Bioassay. The dried bark of *I. megistophylla* (3 kg) was extracted with hot 95% ethanol (10 L) three times, and the solutions were combined and concentrated in vacuo to obtain 150 g of residue. The ethanolic extract was dissolved in water, which was fractionated by liquid−liquid partition with hexanes (three times, each 500 mL), ethyl acetate (six times, each 500 mL), and *n*-butanol (six times, each 500 mL) to yield a hexanes-soluble portion (10 g), an EtOAc-soluble portion (40 g), and an *n*-butanol-soluble portion (50 g), respectively. The different fractions were tested for activities against bacteria, fungi, and herpes simplex virus (HSV-1). The EtOAc fraction showed significant activities against *S. aureus*, *C. albicans*, and herpes simplex virus.

Bioactivity-Guided Isolation. The combined ethyl acetate extracts (40 g) were chromatographed over Si gel (200−400 mesh) eluted with a hexanes−acetone gradient solvent system. Fractions with similar R_f values by TLC were combined to give 42 fractions. All fractions were tested for their activities against bacteria, fungi, and herpes simplex virus (HSV-1). Fractions 7−10 were active against *Staphylococcus aureus*. Fractions 15−24 were active against *S. aureus*, *C. albicans*, and HSV-1. Fractions 7−10 were further separated using a column of Si gel (mesh 240−400) and preparative TLC with a solvent system of hexanes−chloroform (1:1) to afford compounds **1** (52 mg, R_f 0.70), **2** (35 mg, R_f 0.50), and **3** (10 mg, R_f 0.45). Fractions 15−24 were subjected to column chromatography over Si gel eluted with a chloroform−acetone gradient to afford 30 fractions, of which fractions 10−15 were further purified by Sephadex LH-20 (eluted with methanol) and preparative TLC [solvent system hexanes−acetone (3.5:1.5)] to afford **4** (500 mg, R_f 0.71) and **5** (10 mg, R_f 0.70). Fractions 18−20 were passed through the Sephadex LH-20 (eluted with methanol) and purified by preparative TLC [solvent system CH_2Cl_2−acetone−formic acid (3:3:0.1), developed three times], to afford **6** (15 mg, R_f 0.51) and **7** (10 mg, R_f 0.50). Fractions 24−26 were further separated using the Sephadex LH-20 (eluted with methanol) and purified by preparative TLC with the solvent system CH_2Cl_2−methanol−formic acid (3.5:1:0.1) to afford **8** (30 mg, R_f 0.40) and **9** (12 mg, R_f 0.30).

Megislignan (1): amorphous solid; mp 189−190 °C; $[\alpha]_D^{20}$ −18° (*c* 1.0, $CHCl_3$); IR (KBr) ν_{max} 3391, 2935, 1622, 1602, 1508, 1464, 1377, 1232, 1201, 1176, 1130, 1109, 1091, 1031, 1018, 974, 883, 831, 792, 771, 721 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ϵ) 247 (4.42), 284 (4.05), 336 (3.62); ^{13}C and ^1H NMR data, see Table 1; EIMS *m*/*z* 278 (M^+, 100), 263 (15), 247 (8), 231(5), 219 (10), 202 (10), 189 (15), 165 (5); HREIMS *m*/*z* 278.13092, (calcd for $C_{19}H_{18}O_2$ 278.13068).

Megislactone (2): amorphous powder; mp 53−54 °C; $[\alpha]_D^{20}$ −13° (*c* 1.0, $CHCl_3$); IR (KBr) ν_{max} 3529, 3431, 2940, 1759, 1464, 1377, 1350, 1338, 1319, 1257, 1229, 1209, 1195, 1143, 1130, 1051, 985, 717 cm^{-1}; UV (CH_2Cl_2) λ_{max} (log ϵ) 236 (2.67); ^{13}C and ^1H NMR data, see Table 1; EIMS *m*/*z* 478 (M^+, 100), 460 (10), 450 (50), 432 (20), 393 (5), 379 (3), 325 (4), 311(4), 297 (5), 283 (5), 269 (5), 255 (6), 241(5), 227(5), 213(5), 199(3), 195 (5), 185 (3), 181 (6), 171(2), 167 (8), 154 (10), 153 (10), 129 (50), 116 (65), 99 (20), 97 (24), 95 (28), 85 (15), 83 (50), 71 (15), 69 (70), 57 (100); HREIMS *m*/*z* 478.43893 (calcd for $C_{31}H_{58}O_3$ 478.43860).

Acetylation of Megislactone (1). Megislactone (20 mg) was treated with Ac_2O (1 mL) and pyridine (1 mL) overnight at room temperature. Water was added to the mixture, and the suspension was extracted with EtOAc (three times, each 10 mL). The EtOAc extraction was evaporated and crystallized in $CHCl_3$ to afford the acetate **2a** (18 mg) as white powder: EIMS *m*/*z* 520 [M^+] (30), 492 (70), 460 (30), 432 (10), 158 (30), 153 (10), 123 (10), 112 (25), 111 (20), 99 (20), 97 (24), 95 (40), 85 (10), 83 (50), 69 (70), 57 (35), 55 (100).

Elimination of Acetic Acid from Acetate. The acetate **2a** was placed on the top of a column of Al_2O_3 (Merck, activity II/III, 3 g) and eluted with *n*-pentane to give α,β-unsaturated-γ-lactone **2b**. Compound **2b**: amorphous white powder, $[\alpha]_D^{25}$ −25° (*c* 1.0, $CHCl_3$); EIMS *m*/*z* 460 [M^+] (60), 432 (30), 363 (2), 361 (2), 349 (3), 347 (2), 335 (3), 321 (3), 307 (3), 293 (2), 279 (3), 265 (3), 251 (5), 241(6), 237(5), 223(5), 209(3), 195 (5), 181 (6), 167 (8), 154 (5), 153 (10), 139 (10), 125 (8), 112 (30),

111 (20), 99 (20), 97 (24), 95 (40), 85 (10), 83 (50), 71 (25), 69 (70), 57 (40), 55 (100).

Antibacterial and Antifungal Assays. Minimum Inhibitory Concentration (MIC) Determination. MIC values were determined by the broth dilution method using a concentration of 7.0×10^7 colony-forming units/mL of *C. albicans* grown in Sabouraud broth media and 6.0×10^6 colony forming units/mL of *S. aureus* grown in Mueller-Hinton broth media. The MIC was defined as the lowest concentration of substance that prevented growth, which was determined by the appearance of turbidity after 24 h. Solutions of the test compounds were prepared in DMSO (Sigma) and diluted with Saboraud or Mueller-Hinton medium to give final dilutions ranging from 300 to 3 μg/mL. The final concentration of DMSO in the assay did not exceed 2%. The assay was carried out in 96-well microtiter plates. Incubation was at 37 °C for 24 h. Amphotericin B and gentamycin were used as positive controls for *C. albicans* and *S. aureus* with MIC values of 0.055 and 0.1 μg/mL, respectively.[24,25]

Potato Virus X Local Lesion Assay. Compounds to be tested were incubated for 30 min in a PVX solution, which was then used to mechanically infect half-leaves of *Chenopodium quinoa* as described by French et al.[26] The compounds were tested at a final concentration of 9.3 mg/mL in a 5% EGME (ethylene glycol monomethyl ether) solution. All solutions contained a viral concentration of 0.08 mg/mL. Ten μL of solution was applied to each half-leaf. For each leaf used, half was infected with test solution, and half was infected with control solution which contained the same concentration of virus but no inhibitor. Ten days following inoculation, lesions were counted on each half-leaf and the percent inhibition of PVX infection was calculated for each leaf.

Anti-Herpes Simplex Virus (HSV) Assay. Determination of Minimal Inhibitory Concentration (MIC). Assays were carried out with 100 plaque-forming units (PFU) of HSV-1 in Vero cells grown in 96-well culture trays, as described in detail by Hudson et al.[29] The MIC was the minimum concentration of compound that gave complete inactivation of virus infectivity (i.e., absence of viral CPE). A methanolic leaf extract of Adansonia digitata was used as a positive control (minimum antiviral concentration in UVA, 5 μg/mL).[29]

Cytotoxicity Assay. To test for cytotoxicity, Vero cell monolayers were grown in 96-well microtiter plates (Falcon 3072) and exposed to serial dilutions of the corresponding compounds starting at 320 μg/mL. The treated cells were then incubated at 37 °C for 1 h, exposed to UV-A light and visible light for 30 min, and then reincubated for 24 h. The cells were examined microscopically for periodic assessment of changes in cell morphology or visible toxic effects (obvious cellular damage or lysis).

Antiacetylcholinesterase Assay. The effect of compounds **1** and **4** on the rate of the acetylcholinesterase-mediated hydrolysis of acetylthiocholine was determined by measuring the rate of production of free sulfur groups produced as acetylthiocholine is hydrolyzed to thiocholine as described earlier.[30] A flat-bottom 96-well polystyrene cluster plate (300 μL/well) (Corning, Inc.) was used to contain the enzymatic reaction. Absorbency of the colored ion was detected at 414 nm on a Titertek Multiscan machine. In each well the following solutions were added: 10 μL of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co.), 250 μL of 6×10^{-7} M acetylthiocholine iodide (Sigma Chemical Co.), 5 μL of test compound dissolved in methanol at a concentration of 50 mg/mL, 50 μL of 0.5 units/mL of electric eel lyophilized powdered acetylcholinesterase (Sigma Chemical Co.). A 5 μL aliquot of methanol was used for a control. The final concentration of the test compound was 0.8 mg/mL. Each reaction was replicated in eight different wells. The rate of the reaction was calculated, and the percent inhibition was determined as the change between the average rate of control and test reactions.

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