Pycnanthulignenes A−D, Antimicrobial Cyclolignene Derivatives from the Roots of *Pycnanthus angolensis*

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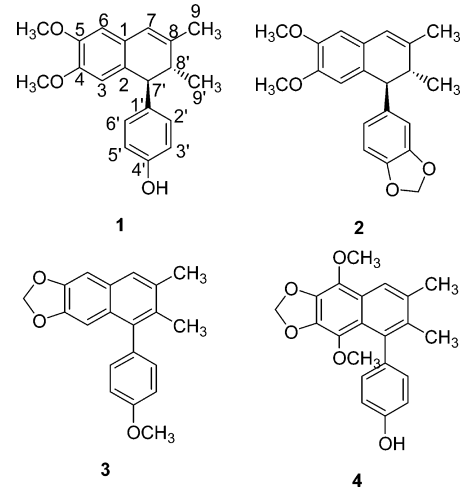
Investigation of the constituents of *Pycnanthus angolensis* roots has resulted in the isolation of four new cyclolignene derivatives, named pycnanthulignene A (**1**), pycnanthulignene B (**2**), pycnanthulignene C (**3**), and pycnanthulignene D (**4**), and six known compounds, 4,5-dimethoxy-3′,4′-methylenedioxy-2,7′-cycloligna-7,7′-diene, 2,7-dimethoxy-3,6-dimethylnaphthalene, 4′-methoxy-4,5-methylenedioxy-2,7′-cyclolign-7-ene, genkwainin, 8-hydroxykanzakiflavone-2, and formononetin. The structures of these compounds were established using spectroscopic methods. Compounds **1** and **3** showed significant antimicrobial activities against a panel of drug-resistant pathogens.

Pycnanthus angolensis (Welw.) Ward (Myristicaceae) is a tree that grows throughout West and Central Africa.[1] Different parts of this plant (leaves, root, wood, and bark) have been used in traditional medicine of Cameroon for treatment of stomach pain,[2] chest pain,[3] and rhinitis problems.[4] This plant is also used to treat malaria,[5] toothache,[6] fungal skin infections,[7] and worms,[8] and some claim a folkloric use for the treatment of leprosy.[8] In earlier work,[9] allantoin was identified as one of the active principles. Phytochemical studies have resulted in the isolation of a wide range of bioactive compounds including dihydroguaiaretic acid,[10] which exhibited antihelmintic and anticancer activity,[5] and pycnanthuquinones A and B,[3,11,12] which presented significant antihyperglycemic activity.[3] Recently, pycnanthuquinone C and other secondary metabolites including lignans and isoflavonoids have been isolated.[7] In order to evaluate the potential of *P. angolensis* as a source of additional bioactive compounds, the root bark of this plant was investigated. In this paper, we report the isolation and structure elucidation of four new cyclolignene derivatives (**1**−**4**) and six known compounds. The in vitro antimicrobial activity of some of these compounds is also reported.



Results and Discussion

Air-dried and ground roots of *P. angolensis* were extracted at room temperature with a mixture of CH_2Cl_2−CH_3OH (1:1). This extract revealed significant antimicrobial activity and was submitted to a bioassay-guided chromatographic fractionation over silica gel as described in the Experimental Section, to afford new cyclolignene derivatives (**1**−**4**), which were named pycnanthulignenes A−D, and six known compounds. The known compounds were identified as 4,5-dimethoxy-3′,4′-methylenedioxy-2,7′-cycloligna-7,7′-diene,[8] 4′-methoxy-4,5-methylenedioxy-2,7′-cyclolign-7-ene,[8] 2,7-dimethoxy-3,6-dimethylnaphthalene,[13] genkwainin,[14] formononetin,[14] and 8-hydroxykanzakiflavone-2[15] by comparison of their spectroscopic data (^{1}H, ^{13}C NMR and MS) with those reported in the literature.

Compound **1**, mp 141−143 °C, was obtained as a colorless powder from a *n*-hexane−AcOEt mixture. Its phenolic nature was confirmed by a positive reaction with ferric chloride. The molecular formula, $C_{20}H_{22}O_3$, was obtained from analysis of its HREIMS, which showed the molecular ion peak $[M]^+$ at *m*/*z* 310.1556 (calcd for $C_{20}H_{22}O_3$, 310.1569), corresponding to 10 degrees of unsaturation. The broadband proton decoupled ^{13}C NMR spectrum (Table 1) of compound **1** showed 17 carbon signals, which were assigned, with the assistance of HSQC and HMBC techniques, as two methoxy groups (two very closely spaced peaks at δ_H 55.9), two methyl groups (δ_C 18.7 and 22.2), seven methines, two of which were sp^3 carbons (δ_C 42.1 and 50.3), and five sp^2 carbons. There were no signals attributable to methylene carbons. The seven remaining ^{13}C signals arose from quaternary carbons including four sp^2 carbons and three oxygenated sp^2 carbons (two at δ_C 147.5 and one at δ_C 153.4).

Analysis of the ^{1}H NMR spectrum (Table 1), combined with the ^{13}C information, indicated that compound **1** contained two aromatic rings, one of which was a *para*-disubstituted benzene ring characterized by signals from an AA′BB′ spin system at δ_H 6.67 (dd, J = 8.5 and 2.5 Hz) and 6.90 (dd, J = 8.5 and 2.5 Hz). The second aromatic ring was a 1,2,4,5-tetrasubstituted benzene, which exhibited a pair of one-proton singlets at δ_H 6.54/δ_C 108.9 and δ_H 6.62/δ_C 114.9 corresponding to two *para* aromatic protons. The other substituents on this ring were two OCH_3 groups that appeared as three-proton singlets at δ_H 3.88/δ_C 55.9 and δ_H 3.78/δ_C 55.9. They were positioned at C-4 and C-5, respectively, using 1D selective NOE experiments, which showed a positive NOE effect on H-3 (δ_H 6.54) when the OCH_3 group at δ_H 3.88 was irradiated and on a proton at δ_H 6.62 (H-6) when the second OCH_3 group at δ_H 3.78 was also irradiated (Figure 1). The ^{1}H NMR spectrum of **1** exhibited a set of signals that consisted of an olefinic singlet at δ_H 6.54/δ_C 108.9, a methyl singlet at δ_H 1.77/δ_C 22.2, a methyl doublet at δ_H 1.07/δ_C 18.7, a methine doublet at δ_H 3.67/δ_C 50.3,

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Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Spectroscopic Data of Compounds **1**−**4**

	1[a]		**2**[a]		**3**[a]		**4**[b]	
Position	δ_C, mult.	δ_H (J in Hz)[c]	δ_C, mult.	δ_H (J in Hz)[c]	δ_C, mult.	δ_H (J in Hz)[c]	δ_C, mult.	δ_H (J in Hz)[c]
1	128.7, qC		128.9, qC		128.7, qC		129.1, qC	
2	127.2, qC		127.0, qC		128.9, qC		129.0, qC	
3	108.9, CH	6.54, s	109.0, CH	6.55, s	102.9, CH	6.63, s	173.7, qC	
4	147.5, qC		147.6, qC		146.5, qC		147.0, qC	
5	147.5, qC		147.7, qC		146.8, qC		146.8, qC	
6	114.9, CH	6.62, s	112.8, CH	6.62, s	113.0, CH	7.04, s	173.3, qC	
7	121.2, CH	6.13, s	121.1, CH	6.13, s	126.4, CH	7.47, s	119.5, CH	6.67, s
7′	50.3, CH	3.67, d (2.9)	50.8, CH	3.65, d (2.9)	137.6, qC		140.0, qC	
8	127.1, qC	139.6, qC	133.6, qC				133.5, qC	
8′	42.1, CH	2.35, dq (7.1, 2.9)	42.2, CH	2.35, dq (7.1, 2.9)	132.9, qC		131.6, qC	
9	22.2, CH_3	1.77, s	22.2, CH_3	1.79, s	17.4, CH_3	2.43, s	35.0, CH_3	2.13, s
9′	18.7, CH_3	1.07, d (7.1)	18.8, CH_3	1.08, d (7.1)	20.9, CH_3	2.09, s	25.7, CH_3	1.73, s
1′	128.7, qC		126.9, qC		131.8, qC		129.8, qC	
2′	138.6, CH	6.90, dd (8.5, 2.5)	138.6, CH	6.52, dd (2.8)	131.1, CH	7.13, dd (8.5, 2.5)	129.1, CH	7.20, dd (8.7, 2.5)
3′	137.7, CH	6.67, dd (8.5, 2.5)	147.4, qC	113.4, CH	7.02, dd (8.5, 2.5)		114.3, CH	6.87, dd (8.7, 2.5)
4′	153.4, qC		145.7, qC		158.5, qC		159.4, qC	
5′	137.8, CH	6.67, dd (8.5, 2.5)	107.8, CH	6.65, d (8.3)	113.8, CH	7.02, dd (8.5, 2.5)	114.2, CH	6.87, dd (8.7, 2.5)
6′	138.7, CH	6.90, dd (8.5, 2.5)	108.1, CH	6.63, dd (8.3, 2.8)	131.9, CH	7.13, dd (8.5, 2.5)	129.6, CH	7.20, dd (8.7, 2.5)
3-OCH_3							55.1, CH_3	3.32, s
4-OCH_3	55.9, CH_3	3.88, s	55.8, CH_3	3.79, s				
5-OCH_3	55.9, CH_3	3.78, s	55.8, CH_3	3.88, s				
6-OCH_3							55.1, CH_3	3.35, s
4′-OCH_3					55.3, CH_3	3.89, s		
4′-OH		7.26, s						6.62, s
$-O_2CH_2-$			100.7, CH_2	5.87, d (3.2)	100.7, CH_2	5.94, s	100.7, CH_2	5.31, d (2.6)

[a] In $CDCl_3$. [b] In C_6D_6. [c] Chemical shifts are displayed in δ (ppm) downfield from TMS; coupling constants are in Hz. The couplings in AA′BB′ spin systems were obtained by simulation analysis using the Spinworks program.

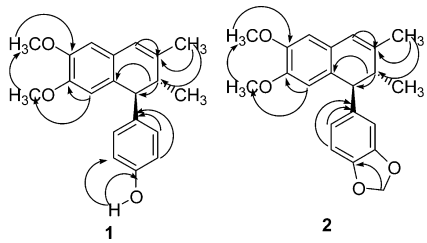

Figure 1. Relevant HMBC correlations of compounds **1** and **2**.

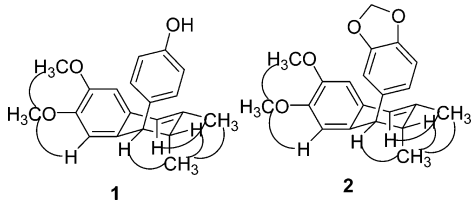

Figure 2. Relevant NOE correlations (NOESY data) of compounds **1** and **2**.

and a methine multiplet at δ_H 2.35/δ_C 42.1, which were attributed on the basis of COSY and HMBC spectra to a 2,3-dimethylbut-1-enyl moiety. The HMBC spectrum of **1** also showed cross-peaks linking the methine doublet at δ_H 3.67 (H-7′) to the carbon at δ_C 127.2 (C-2) belonging to the 1,2,4,5-tetrasubstituted benzene system, indicating that the 2,3-dimethylbut-1-enyl moiety and the 1,2,4,5-tetrasubstituted benzene ring formed a dihydronaphthalene skeleton on which the *para*-disubstituted benzene moiety was linked at position C-7′. This was confirmed by the HMBC spectrum, which showed correlation peaks of the proton at δ_H 3.67 (H-7′) with carbons at δ_C 128.7 (C-1) and δ_C 138.6 (C-2) (Figure 1). The relative configuration of substituents on the dihydronaphthalene ring was established using 1D selective NOE experiments, which showed a positive NOE effect on methyl protons on C-9′, when H-7′ was irradiated, indicating that H-7′ and the methyl at C-8′ were close spatially (Figure 2). This result, in addition to the lower coupling constants between H-7′ (δ_H 3.67, d, J = 2.9 Hz) and H-8′ (δ_H 2.35, dq, J = 7.1 and 2.9 Hz), led to the conclusion that the relative configuration of these the two protons was equatorial−equatorial. The combination of COSY, HSQC, and HMBC experiments permitted assignment of all ^{13}C NMR signals of compound **1** (Table 1). Thus, the structure of **1** (pycnanthulignene A) was established to be 4,5-dimethoxy-2,7′-cyclolign-7-en-4′-ol.

Compound **2** was obtained as a yellow, viscous oil. Its molecular formula, $C_{21}H_{22}O_4$, was deduced from its HREIMS at m/z 338.1517 (calcd for $C_{21}H_{22}O_4$, 338.1518). The ^{1}H and ^{13}C NMR data (Table 1) were very close to those of compound **1**, indicating that **2** was also a cyclolignene derivative. Comparison of the spectroscopic data of **1** and **2** revealed that the only difference between them was the level of substitution of the aromatic ring attached to C-7′. In compound **1** this benzene ring was *para*-disubstituted. In compound **2** the AA′BB′ spin system was replaced by an ABX spin system of three aromatic protons at δ_H 6.52 (d, J = 2.8 Hz, H-2′), δ_H 6.63 (dd, J = 8.3 and 2.8 Hz, H-6′), and δ_H 6.65 (d, J = 8.3 Hz, H-5′) and by a two-proton singlet at δ_H 5.87/δ_C 100.7 characteristic of a methylenedioxy moiety. Thus, compound **2** (pycnanthulignene B) was elucidated to be 4,5-dimethoxy-3′,4′-methylenedioxy-2,7′-cyclolign-7-ene.

Compound **3**, mp 158−160 °C, had the molecular formula $C_{20}H_{18}O_3$ (HREIMS). This molecular formula, when compared to that of compound **1**, indicated that **3** had two more degrees of unsaturation than **1**. Comparison of their ^{1}H NMR spectra showed many similarities, including the presence of a pair of *para*-aromatic protons at δ_H 6.63 (H-3) and δ_H 7.04 (H-6) in **3**, an AA′BB′ spin system corresponding to a *para*-substituted benzene ring, and two three-proton singlets due to methyl groups linked to two sp^2 carbons. The difference between the two compounds was the absence of signals corresponding to two OCH_3 groups in **3** and their replacement by a methylenedioxy moiety that appeared at δ_H 5.94/δ_C 100.7. The ^{1}H NMR spectrum also showed an aromatic singlet at δ_H 7.47 (H-7) and an OCH_3 group [C-4′ (δ_C 158.5)]. The absence of signals that appeared at δ_H 3.67 (H-7′) and δ_H 6.13 (H-7) in compound **1** and the downfield shift of two methyl groups suggested that the two aliphatic protons were replaced by a double bond, leading to a cycloligna-7,7′-diene.[16] The combination of COSY, HSQC, and HMBC experiments permitted complete assignments of the ^{13}C

Table 2. Antimicrobial Activities of Compounds **1** and **3** and Reference Antibiotics (RA)

	MIC[a(b)]			MMC[a(b)]		
microorganism[c]	**1**	**3**	RA[d]	**1**	**3**	RA[d]
Bacteria						
MRSA	9.76 (28.7)	19.53 (63.8)	4.88 (0.9)	19.53 (57.7)	39.1 (128.0)	9.8 (1.8)
βL$^+$EC	19.5 (57.7)	19.5 (63.8)	4.88 (0.9)	39.1 (115.0)	78.1 (255.1)	9.8 (1.8)
βL$^+$SD	39.1 (115.5)	39.1 (127.6)	4.88 (0.9)	78.1 (230.9)	>78.1 (>255.1)	9.8 (1.8)
ARKP	78.12 (230.9)	78.12 (255.1)	19.53 (3.6)	>78.12 (>230.9)	>78.1 (>255.1)	19.5 (3.6)
CRPA	78.12 (230.9)	>78.12 (>255.1)	9.76 (1.8)	>78.12 (>230.9)	nd[e]	19.5 (3.6)
CRST	19.53 (57.7)	39.06 (127.6)	9.76 (1.8)	39.06 (115.5)	78.12 (255.1)	19.5 (3.6)
CRCF	39.06 (115.5)	39.06 (127.6)	4.88 (0.9)	78.12 (230.9)	78.12 (255.1)	9.8 (1.8)
Fungi						
C. albicans	19.53 (57.7)	19.53 (63.8)	4.88 (0.5)	39.06 (115.5)	39.06 (127.6)	9.8 (1.0)
M. audouinii	39.06 (115.5)	39.06 (127.6)	4.88 (0.5)	78.12 (230.9)	>78.1 (>255.1)	9.8 (1.0)

[a] In μg/mL. [b] In μmol. [c] The tested microorganisms are MRSA (methicillin-resistant *Staphylococcus aureus*), βL$^+$EC (β-lactamase positive *Escherichia coli*), βL$^+$SD (β-lactamase positive *Shigella dysenteriae*), ARKP (ampicillin-resistant *Klebsiella pneumoniae*), CRPA (carbenicillin-resistant *Pseudomonas aeruginosa*), CRST (chloramphenicol-resistant *Salmonella typhi*), CRCF (chloramphenicol-resistant *Citrobacter freundii*), *C. albicans* (*Candida albicans*), *M. audouinii* (*Microsporum audouinii*). [d] Gentamicin and nystatin were used as references antibiotics (RA) for bacteria and fungi, respectively. [e] nd: not determined because MIC > 78.12 μg/mL.

NMR signals of **3**. Thus, the structure of **3** (pycnanthulignene C) was established as 4′-methoxy-4,5-methylenedioxy-2,7′-cycloligna-7,7′-diene.

Compound **4**, mp 164.5−165.5 °C, had the molecular formula $C_{21}H_{20}O_5$. Comparison of its ^{1}H and ^{13}C NMR data to those of **3** revealed that **4** was also a cycloligna-7,7′-diene. The main difference between the two compounds was the substitution pattern of their arylnaphthalene rings. In compound **4**, the pair of *para*-aromatic protons that resonated at δ_H 6.63 (H-3) and δ_H 7.04 (H-6) in **3** were replaced by a pair of OCH_3 groups that appeared at δ_H 3.32/δ_C 55.1 (C-3) and δ_H 3.35/δ_C 55.1 (C-6). Furthermore, the OCH_3 group, which in compound **3** occupied the C-4′ position, was replaced in compound **4** by an OH group. Thus, compound **4** (pycnanthulignene D) was 3,6-dimethoxy-4,5-methylenedioxy-2,7′-cycloligna-7,7′-dien-4′-ol.

Compounds **1** and **3** were evaluated in vitro for antimicrobial activity against a panel of drug-resistant bacteria and pathogenic fungi strains. Among the drug-resistant strains, there were one Gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA, LMP805), and six Gram-negative bacteria including β-lactamase positive (βL$^+$) *Escherichia coli* (βL$^+$EC, LMP701), (βL$^+$) *Shigella dysenteriae* (βL$^+$SD, LMP606), ampicillin-resistant *Klebsiella pneumoniae* (ARKP, LMP803), carbenicillin-resistant *Pseudomonas aeruginosa* (CRPA, LMP804), chloramphenicol-resistant *Salmonella typhi* (CRST, LMP706), and chloramphenicol-resistant *Citrobacter freundii* (CRCF, LMP802). The two pathogenic fungi used were *Candida albicans* (*C. albicans*, LMP709U) and *Microsporum audouinii* (*M. audouinii*, LMP725D). Gentamicin and nystatin were used as references for antibacterial and antifungal tests, respectively. Compound **1** (Table 2) had significant activity against all of the tested organisms, methicillin-resistant *S. aureus* being the most sensitive pathogen. The minimal inhibitory concentration (MIC) values for **1** varied from 28.7 μM (against *S. aureus*) to 230.9 μM (vs *K. pneumoniae* and *P. aeruginosa*). Compound **3** also exhibited noteworthy activity against eight of the nine tested microorganisms. The lowest MIC values (63.8 μM) observed with this compound were against *S. aureus*, *E. coli*, and *C. albicans*. Although compounds **1** and **3** were somewhat less active than the reference antibiotics, the fact that they showed micromolar inhibitory potential against these drug-resistant microorganisms is noteworthy. Also, many of the minimal microbicidal concentration (MMC) values obtained with the test compounds were 4-fold less than their corresponding MIC values (Table 2).

Experimental Section

General Experimental Procedures. Melting points were recorded on a Gallenkamp melting point apparatus and were not corrected, optical rotations were recorded on an Autopol V automatic polarimeter, ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz in $CDCl_3$ or C_6D_6, respectively, HREIMS were run on a VG7070E-HF mass spectrometer, and TLC analyses were performed on Kieselgel 60F254 precoated Al sheets (0.2 mm layer thickness, Merck) with spots being detected by UV radiation (254 or 366 nm) and staining with 10% (v/v) H_2SO_4.

Plant Material. Roots of *Pycnanthus angolensis* were collected in April 2007 at Ezezang, in the center province of the Republic of Cameroon, and were identified by M. Nana (plant taxonomist) of the National Herbarium, Yaounde, Cameroon. A voucher specimen of the plant is deposited in the National Herbarium, Yaounde, Cameroon (No. 19536/SRFCam).

Extraction and Isolation. Air-dried and powdered root bark of *P. angolensis* (2.7 kg) was exhaustively extracted at room temperature for 70 h using a mixture of CH_2Cl_2−CH_3OH (1:1). The suspension was filtered, and the filtrate was concentrated under reduced pressure to give 180 g of brown residue. This residue was then subjected to vacuum column chromatography (CC) on silica gel (Merck, 230−400 mesh), eluting with *n*-hexane, *n*-hexane−EtOAc (4:1), *n*-hexane−EtOAc (1:1), and EtOAc to give four fractions: A (30 g), B (33 g), C (34 g), and D (40 g). Fraction A was subjected to CC on silica gel (Merck 70−230 mesh) and eluted with *n*-hexane−EtOAc mixtures of increasing polarity. One hundred fractions of 100 mL each were collected and analyzed by TLC using *n*-hexane−CH_2Cl_2 (9:1). Evaporation of the solvent afforded a residue in each case, and these residues were further separated by CC and TLC to provide **2** (46 mg), **4** (26 mg), 2,7-dimethoxy-3,6-dimethylnaphthalene (32.5 mg), and 4′-methoxy-4,5-methylenedioxy-2,7′-cyclolign-7-ene (29.6 mg). Fraction B was subjected to repeated silica gel CC eluting with *n*-hexane−EtOAc mixtures of increasing polarity to give **1** (800 mg), **3** (44.6 mg), 8-hydroxykanzakiflavone-2 (37.8 mg), and formononetin (16.2 mg). Finally, repeated silica gel CC of fraction C eluting with *n*-hexane−EtOAc mixtures of increasing polarity furnished 4,5-dimethoxy-3′,4′-methylenedioxy-2,7′-cycloligna-7,7′-diene (79 mg) and genkwainin (25 mg).

Pycnanthulignene A (1): white powder ($CDCl_3$); mp 141−143 °C; $[\alpha]_D^{20.2}$ +33.8 (*c* 0.31, EtOH); ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 310 $[M]^+$ (100), 279 (14), 295 (62), 264 (10), 217 (8), 202 (6), 107 (5); HREIMS *m*/*z* 310.1556 (calcd for $C_{20}H_{22}O_3$, 310.1569).

Pycnanthulignene B (2): yellow. viscous oil ($CDCl_3$); $[\alpha]_D^{20.2}$ +118.5 (*c* 0.338, EtOH); ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 338 $[M]^+$ (100), 323 (56), 307 (13), 261 (8), 217 (8), 202 (12), 147 (12); HREIMS *m*/*z* 338.1517 (calcd for $C_{21}H_{22}O_4$, 338.1518).

Pycnanthulignene C (3): white powder ($CDCl_3$); mp 158−160 °C; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 306 $[M]^+$ (100), 291 (10), 260 (4), 230 (8), 189 (12); HREIMS *m*/z 306.2248 (calcd for $C_{20}H_{18}O_3$, 306.2256).

Pycnanthulignene D (4): brown crystals (C_6D_6); mp 164.5−165 °C; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 352 $[M]^+$ (100), 334 (60), 322 (15), 290 (26), 259 (12); HREIMS 352.1312 (calcd for $C_{21}H_{20}O_5$, 352.1311).

Biological Testing. Compounds **1** and **3** were tested for their antimicrobial effects against a series of microorganisms using a microbroth dilution method according to the protocol described in the

literature.[17] The assay was repeated three times. The MIC of samples was determined following addition of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (40 μL) and incubating at 37 °C for 30 min. Viable organisms reduced the yellow dye to a pink color. The MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth. For determination of the MMC, a portion of liquid (5 μL) from each well that showed no change in color was plated on MHA and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth after this subculturing was taken as the MMC.[17]

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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