Microbial Transformation of (−)-Guaiol and Antibacterial Activity of Its Transformed Products

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Microbial transformation of the sesquiterpene (−)-guaiol (**1**) [1(5)-guaien-11-ol] was investigated using three fungi, *Rhizopus stolonifer*, *Cunninghamella elegans*, and *Macrophomina phaseolina*. Fungal transformation of **1** with *Rhizopus stolonifer* yielded a hydroxylated product, 1-guaiene-9β,11-diol (**2**). In turn, *Cunninghamella elegans* afforded two mono- and dihydroxylated products, 1-guaiene-3β,11-diol (**3**) and 1(5)-guaiene-3β,9α,11-triol (**4**), while *Macrophomina phaseolina* produced two additional oxidative products, 1(5)-guaien-11-ol-6-one (**5**) and 1-guaien-11-ol-3-one (**6**). All metabolites were found to be new compounds as deduced on the basis of spectroscopic techniques. Compounds **1**−**6** were evaluated for their activity against several bacterial strains.

(−)-Guaiol (**1**), $C_{15}H_{26}O$, a sesquiterpene alcohol having the guaiane skeleton, found in many medicinal plants,[1] has been isolated from *Guaiacum* wood oil of the tree *Bulnesia sermienti*. It is a key compound in the biogenesis of a large number of guaiane natural products.[2] The essential oils of *Salvia lanigera*[3] and *Helitta longifoliata*,[4] which both contain compound **1** as a major component, were found to possess pronounced antibacterial activity.

Structural modifications of naturally occurring bioactive substances by microbial transformation, which are otherwise not possible by conventional chemical methods, is an important area of natural product chemistry. We have reported previously the microbial transformation of several bioactive sesquiterpenes such as (−)-ambrox, (+)-sclareolide, (+)-isolongifolene, (+)-isolongifolol, and sclareol.[5−8] In continuation of our studies on the microbial transformation of bioactive compounds, the sesquiterpene (−)-guaiol (**1**) has been incubated with *Rhizopus stolonifer*, *Cunninghamella elegans*, and *Macrophomina phaseolina*, which afforded metabolites **2**−**6**. (−)-Guaiol (**1**) was subjected to microbial transformation with the aim of producing structurally modified compounds with more pronounced antibacterial activity.

From small-scale experiments it was shown that the fungi *Rhizopus stolonifer*, *Cunninghamella elegans*, and *Macrophomina phaseolina* are capable of converting (−)-guaiol (**1**) into more oxidized metabolites. Preparative-scale fermentation was thus carried out to produce sufficient quantities of the metabolites for structure elucidation and biological studies. Incubation of (−)-guaiol (**1**) with *Rhizopus stolonifer* yielded metabolite **2**. When **1** was incubated with *Cunninghamella elegans*, mebabolites **3** and **4** were obtained. Incubation of (−)-guaiol (**1**) with *Macrophomina phaseolina* yielded metabolites **5** and **6**. The structures of new metabolites **2**−**6** were elucidated through spectroscopic studies. Compound **2** was obtained as an amorphous solid. The HREIMS exhibited a molecular ion [M^+] peak at *m*/*z* 238.1574, corresponding to the formula $C_{15}H_{26}O_2$ (calcd 238.1583), in accord with a monohydroxy derivative of **1**. The ^{1}H NMR spectrum of compound **2** showed downfield signals for an oxygen-bearing methine proton at δ 3.87 (ddd, $J_{9\alpha,10\alpha}$ = 11.3 Hz, $J_{9\alpha,8\beta}$ = 7.7 Hz, $J_{9\alpha,8\alpha}$ = 3.9 Hz) and an olefinic proton at δ 5.47 (t, J = 5.8 Hz), which were correlated with the carbon signals at δ 76.6 and 124.9, respectively, in the HMQC spectrum. The ^{13}C NMR spectrum revealed the presence of 15 carbons, including four methyls, three methylenes, six methines, and three quaternary carbons. A downfield signal at δ 3.87 was assigned to H-9 on the basis of COSY 45° interactions between H-9 and the C-8 methylene protons (δ 2.23, 2.03), as well as HMBC correlations between the C-8 methylene protons (δ 2.23, 2.03) and C-9 (δ 76.6). Another downfield methine proton, appearing at δ 5.47, was assigned to olefinic H-2. The migration of a double bond from C-1/C-5 in **1** to C-1/C-2 in **2** was inferred from an olefinic methine carbon signal at δ 124.9. The COSY 45° interactions between H-2 (δ 5.47) and the C-3 methylene protons (δ 2.20, 1.20) further supported the migration of the double bond. The configurations of the new stereogenic centers were deduced by a NOESY experiment (Figure 1). From these data, the structure of metabolite **2** was deduced as 1-guaiene-9β,11-diol.

Compound **3** was isolated as colorless needles. Its HREIMS exhibited a M^+ peak at *m*/*z* 238.1530, corresponding to the formula $C_{15}H_{26}O_2$ (calcd 238.1553), 16 amu higher than the parent compound, **1**. The ^{1}H NMR spectrum of compound **3** showed two downfield methine proton signals, characteristic of a hydroxyl and double bond, at δ 4.51 (dd, $J_{3\alpha,2}$ = 6.5 Hz, $J_{3\alpha,4\alpha}$ = 4.2 Hz) and 5.41 (d, $J_{2,3\alpha}$ = 6.5 Hz), respectively. The ^{13}C NMR spectrum showed resonances for two new methine signals at δ 81.3 (C-3), and 127.8 (C-2). COSY 45° interactions between H-3 (δ 4.51) and H-2 (δ 5.41) and H-4 (δ 1.99) and the HMBC correlations between H-2 and C-3 (δ 81.3) and C-4 (δ 44.5) indicated the presence of the hydroxyl group at C-3. The position of double bond in compound **3** was the same as in compound **2**. NOESY correlations were used to assign the configurations at C-3 and C-5 (Figure 1). Thus, the structure of metabolite **3** was deduced as 1-guaiene-3β,-11-diol.

Compound **4** was isolated as a white, crystalline solid. The HREIMS exhibited an M^+ peak at *m*/*z* 254.1407, corresponding to the formula $C_{15}H_{26}O_3$ (calcd 254.1426), indicating two additional OH groups, relative to the parent compound **1**. The ^{1}H NMR spectrum of compound **4** displayed two new methine signals at δ 3.49 (dd, $J_{9\beta,8\alpha}$ = 6.5 Hz, $J_{9\beta,10\alpha}$ = 4.5 Hz) and 3.87 (dt, $J_{3\alpha,4\alpha}$ = 11.1 Hz, $J_{3\alpha,2\alpha}$ = 11.1 Hz, $J_{3\alpha,2\beta}$ = 5.8 Hz), characteristic of protons geminal to hydroxyl groups. The ^{13}C NMR spectrum showed two downfield methine carbon signals at δ 61.6 (C-9) and 76.2 (C-3). COSY 45° interactions between H-3 (δ 3.87) and the C-2 methylene protons (δ 2.56, 1.20) indicated the position of the hydroxyl group at C-3. In the HMBC spectrum, H-9 (δ 3.49) showed heteronuclear interactions with C-10 (δ 40.1) and indicated a hydroxyl group at C-9. The configurations of the hydroxyl groups at C-3 and at C-9 were assigned as β and α, respectively, on the basis of NOESY correlations (Figure 1). Compound **4** was thus deduced as 1(5)-guaiene-3β,9α,11-triol.

Compound **5** was isolated as a white, crystalline solid. The HREIMS of compound **5** showed an M^+ peak at *m*/*z* 236.1238 ($C_{15}H_{24}O_2$, calcd 236.1247). The absorption band at 1687 cm^{-1} in the IR spectrum and the absorption at 238 nm in UV spectrum

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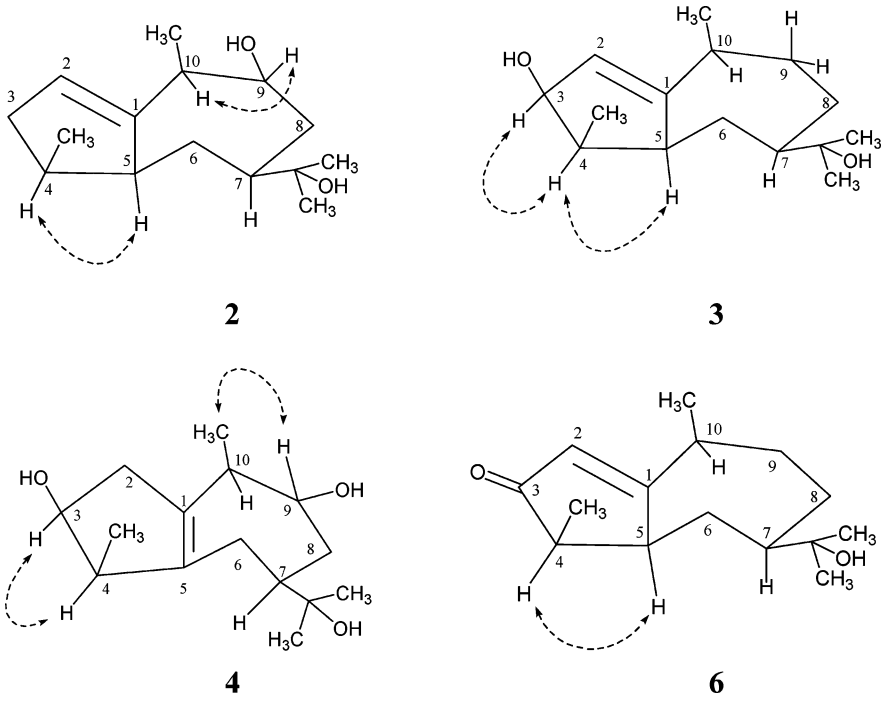
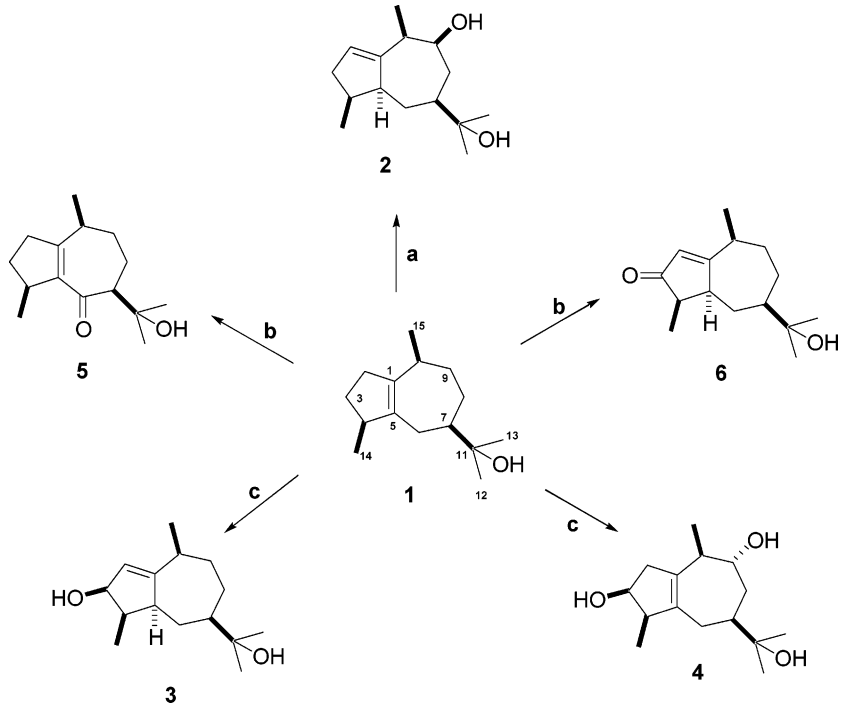


2 **3** **4** **6**

Figure 1. Key NOESY correlations in compounds **2**−**4** and **6**.

Scheme 1. Transformation of (−)-Guaiol (**1**) with (a) *Rhizopus stolonifer*, (b) *Macrophomina phaseolina*, and (c) *Cunninghamella elegans*



were reflective of the presence of a conjugated system. The ^{1}H NMR spectrum of compound **5** was very similar to that of compound **1**, while the ^{13}C NMR spectrum displayed an additional quaternary signal at δ 208.1, characteristics of a ketonic carbonyl group. The position of the ketone was inferred at C-6 on the basis of the downfield shift of C-5 (δ 177.3) and the HMBC correlations of H-7 (δ 2.32) with C-6 and C-11. The structure of the metabolite **5** was thus deduced as 1(5)-guaien-11-ol-6-one.

Compound **6** was isolated as a crystalline solid. The HREIMS exhibited an M^+ peak at *m*/*z* 236.1389, corresponding to the formula $C_{15}H_{24}O_2$ (calcd 236.1374). The IR spectrum exhibited an absorption band at 1739 cm^{-1}, indicating the presence of a carbonyl group in the molecule. The ^{1}H NMR spectrum of compound **6** showed an additional downfield signal at δ 5.72, which was correlated with the methine carbon resonating at δ 127.2 in the HMQC spectrum. The ^{13}C NMR spectrum showed the presence of 15 carbons including four methyls, three methylenes, five methines, and three quaternary carbons. The migration of a double bond from C-1/C-5 to C-1/C-2 and the introduction of a carbonyl group were inferred from the allylic COSY 45° interactions between H-2 (δ 5.72) and H-10 (δ 1.56) and the HMBC correlations of H-2 with C-3 (δ 209.1) and C-10 (δ 34.8). The configuration at the new stereogenic center

Table 1. ^{1}H NMR Data (400 MHz, $CDCl_3$) for Compounds **2**−**6**

position	**2** δ_H (*J* in Hz)	**3** δ_H (*J* in Hz)	**4** δ_H (*J* in Hz)	**5** δ_H (*J* in Hz)	**6** δ_H (*J* in Hz)
2	5.47, t (5.8)	5.41, d (6.5)	2.56, 1.20, m	2.52, 1.24, m	5.72, s
3	2.20, 1.20, m	4.51, dd (6.5, 4.2)	3.87, dt (11.1, 11.1, 5.8)	2.10, 1.32, m	
4	1.29, m	1.99, m	1.95, m	1.28, m	2.01, m
5	1.70, m	1.68, m			1.71, m
6	2.25, 1.59, m	2.23, 1.62, m	2.26, 1.54, m		2.20, 1.60, m
7	1.82, m	1.93, m	1.80, m	2.32, m	1.89, m
8	2.23, 2.03, m	1.82, 1.80, m	2.34, 2.06, m	2.01, 1.20, m	1.84, 1.80, m
9	3.87, ddd (11.3, 7.7, 3.9)	1.93, 1.50, m	3.49, dd (6.5, 4.5)	1.94, 1.56, m	1.92, 1.54, m
10	1.92, m	1.55, m	1.92, m	1.54, m	1.56, m
12	1.20, s	1.21, s	1.19, s	1.19, s	1.20, s
13	1.23, s	1.25, s	1.23, s	1.25, s	1.24, s
14	1.16, d (6.7)	1.04, d (6.7)	0.88, d (6.7)	1.12, d (6.7)	1.08, d (6.7)
15	1.04, d (6.8)	1.14, d (6.8)	0.90, d (6.8)	0.97, d (6.8)	1.14, d (6.8)

Table 2. ^{13}C NMR Data (100 MHz, $CDCl_3$) for Compounds **2**−**6**

position	**2** δ_C, mult.	**3** δ_C, mult.	**4** δ_C, mult.	**5** δ_C, mult.	**6** δ_C, mult.
1	152.0, q,C	155.0, qC	140.0, Qc	145.3, qC	158.0, Qc
2	124.9, CH	127.8, CH	36.0, CH_2	38.0, CH_2	127.2, CH
3	37.6, CH_2	81.3, CH	76.2, CH	32.8, CH_2	209.1, qC
4	42.4, CH	44.5, CH	45.4, CH	42.9, CH	46.1, CH
5	33.2, CH	33.0, CH	138.8, qC	177.3, qC	32.5, CH
6	32.2, CH_2	31.0, CH_2	32.0, CH_2	208.1, qC	31.9, CH_2
7	51.0, CH	52.5, CH	50.7, CH	59.8, CH	51.1, CH
8	41.6, CH_2	38.2, CH_2	44.4, CH_2	36.6, CH_2	35.9, CH_2
9	76.6, CH	33.2, CH_2	61.6, CH	31.9, CH_2	31.4, CH_2
10	42.2, CH	34.6, CH	40.1, CH	34.1, CH	34.8, CH
11	73.9, qC	73.8, qC	73.0, qC	73.3, qC	73.4, qC
12	26.5, CH_3	26.6, CH_3	26.9, CH_3	25.7, CH_3	24.7, CH_3
13	28.9, CH_3	28.8, CH_3	28.2, CH_3	28.0, CH_3	27.9, CH_3
14	20.2, CH_3	14.4, CH_3	18.6, CH_3	19.3, CH_3	19.4, CH_3
15	13.0, CH_3	20.2, CH_3	12.6, CH_3	22.8, CH_3	22.5, CH_3

C-5 was assigned as α on the basis of NOESY correlations (Figure 1). Thus, the structure of metabolite **6** was deduced as 1-guaien-11-ol-3-one.

Compounds **1**−**6** were screened for antibacterial activity[9] against six bacterial strains, i.e., *Escherichia coli*, *Bacillus subtilis*, *Shigella flexneri*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella typhi*, and the results are shown in Table 3. Preliminary results indicated that compounds **2** and **3**, resulting from the migration of the double bond from C-1/C-5 to C-1/C-2 and the hydroxylation at various position, showed more potent activity against *Pseudomonas aeruginosa* than the parent compound **1** (20 mm vs 17 mm inhibition zone diameter). Similarly, compound **4**, with two additional hydroxyl groups, as compared to the substrate **1**, showed activity against *Staphylococcus aureus* (20 mm zone of inhibition at 100 μg/100 μL). Imipenem, a broad-spectrum β-lactam antibiotic, was used as a standard. Interestingly substrate **1** was found to be as active as the control (imipenem) with comparable zones of inhibition (Table 3).

Experimental Section

General Experimental Procedures. Melting points were determined with a Buchi-535 melting point apparatus. Optical rotations were measured with a JASCO DIP-360 digital polarimeter. UV spectra were recorded with a Hitachi U-3200 spectrophotometer. IR spectra were obtained in KBr with a Shimadzu 8900 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance-400 NMR spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) in $CDCl_3$. The chemical shifts (δ) in parts per million (ppm) were in relation to TMS as an internal standard. The coupling constants (*J*) are reported in hertz (Hz). EIMS and HREIMS were obtained with a JEOL JMS-600H mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel precoated plates (Merck, PF_{254}, 20 × 20, 0.25 mm). Column chromatography was carried out on silica gel (70−230 mesh, Merck). The compounds were detected on TLC using vanillin spray reagent. (−)-Guaiol (**1**) was purchased from Fluka (Buchs, Switzerland).

Organisms. The microbial cultures were originally obtained from the American Type Culture Collection (ATCC), Northern Regional Research Laboratories (NRRL), and Karachi University Culture Collection (KUCC). Fungal cultures of *Rhizopus stolonifer* (ATCC 10404), *Cunninghamella elegans* (NRRL 1392), and *Macrophomina phaseolina* (KUCC 730) were grown on Sabouraud 4% glucose agar (Merck) at 25 °C and stored at 4 °C.

General Fermentation and Extraction Conditions. The screening experiments were carried out as reported before[5−10] and according to a standard two-stage protocol.[11] The substrate **1** was dissolved in acetone, and the resulting clear solution was evenly distributed among 30 flasks (20 mg/0.5 mL in each flask), containing 24-h-old stage II cultures. Fermentation was carried out for several days on a rotatory shaker (200 rpm) at 29 °C. During the fermentation period, aliquots from the culture were taken out daily and analyzed by TLC in order to determine the degree of transformation of substrate. In all experiments, one control flask without fungus (for checking substrate stability) and another flask with exogenous substrate (for checking endogenous metabolites) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with CH_2Cl_2 (1.5 L), and the filtrate was extracted with CH_2Cl_2 (3 × 2 L). The combined organic extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and analyzed by TLC. Control flasks were also harvested and compared with the test by TLC to confirm the presence of biotransformed products.

Fermentation of (−)-Guaiol (1) with *Rhizopus stolonifer*. (−)-Guaiol (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing a 24-h-old stage II culture. Fermentation was carried out for 12 days. After filtration, extraction, and evaporation, a brown gum (1.1 g) was obtained, which, on repeated

Table 3. Antibacterial Activities for Compounds **1**−**6** against Gram-positive Bacteria[a]

compound	*Escherichia coli*	*Bacillus subtilis*	*Shigella flexneri*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Salmonella typhi*
1	16	15	17		17	24
2					20	16
3					20	15
4				20	17	15
5		11	10		13	
6		11			13	
imipenem[b]	30	33	27	33	24	25

[a] Drug concentration: 100 μg/100 μL. Inhibition zones are given in mm. [b] Positive control (100 μg/100 μL).

column chromatography (petroleum ether−AcOEt gradient), yielded compound **2** (10 mg; with petroleum ether−AcOEt, 83:17).

1-Guaiene-9β,11-diol (2): amorphous solid; $[\alpha]^{25}_D$ −64 (*c* 0.2, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 204 (2.41) nm; IR ($CHCl_3$) ν_{max} 3451, 1460, 1142 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 238 $[M]^+$ (3), 220 (60), 202 (22), 161 (11), 149 (100); HREIMS *m*/*z* 238.1574 (calcd for $C_{15}H_{26}O_2$, 238.1583).

Fermentation of (−)-Guaiol (1) with *Cunninghamella elegans*. (−)-Guaiol (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing a 24-h-old stage II culture. Fermentation was carried out for 12 days. After filtration, extraction, and evaporation, a brown gum (1.3 g) was obtained, which, on repeated column chromatography (petroleum ether−AcOEt gradient), yielded metabolites **3** (11 mg; with petroleum ether−AcOEt, 82:18) and **4** (11 mg; with petroleum ether−AcOEt, 81:19).

1-Guaiene-3β,11-diol (3): colorless needles; mp 105−108 °C; $[\alpha]^{25}_D$ −105 (*c* 0.2, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 202 (3.10) nm; IR ($CHCl_3$) ν_{max} 3370, 1554, 1024 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 238 $[M]^+$ (5), 220 (10), 202 (13), 161 (7), 149 (100); HREIMS *m*/*z* 238.1530 (calcd for $C_{15}H_{26}O_2$, 238.1553).

1(5)-Guaiene-3β,9α,11-triol (4): white, crystalline solid; mp 103−107 °C; $[\alpha]^{25}_D$ −118 (*c* 0.3, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 205 (2.63) nm; IR ($CHCl_3$) ν_{max} 3375, 2926, 1664, 1044 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 254 $[M]^+$ (9), 236 (7), 218 (16), 200 (4), 195 (4), 109 (100), 57 (44), 124 (66); HREIMS *m*/*z* 254.1407 (calcd for $C_{15}H_{26}O_3$, 254.1426).

Fermentation of (−)-Guaiol (1) with *Macrophomina phaseolina*. Compound **1** (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing a 24-h-old stage II culture. Fermentation was carried out for 12 days. After filtration, extraction, and evaporation, a brown gum (1.7 g) was obtained, which, on repeated column chromatography (petroleum ether−AcOEt gradient), yielded compounds **5** (12 mg; with petroleum ether−AcOEt, 89:11) and **6** (13 mg; with petroleum ether−AcOEt, 88:12).

1(5)-Guaien-11-ol-6-one (5): white, crystalline solid; mp 89−91 °C; $[\alpha]^{25}_D$ −81 (*c* 0.3, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 238 (3.70) nm; IR ($CHCl_3$) ν_{max} 3435, 1633, 1687 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 236 $[M]^+$ (9), 218 (23), 200 (25), 161 (41), 149 (100); HREIMS *m*/*z* 236.1238 (calcd for $C_{15}H_{24}O_2$, 236.1247).

1-Guaien-11-ol-3-one (6): crystalline solid; mp 102−104 °C; $[\alpha]^{25}_D$ −113 (*c* 0.3, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 240 (4.31) nm; IR ($CHCl_3$) ν_{max} 3449, 1653, 1739 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 236 $[M]^+$ (19), 218 (42), 200 (61), 161 (54), 149 (100); HREIMS *m*/*z* 236.1389 (calcd for $C_{15}H_{24}O_2$, 236.1374).

Antibacterial Activity. All metabolites were screened against *Bacillus subtilis*, *Staphylococcus aureus*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli* strains.[8] For antibacterial screening, 3 mg of each sample was taken and dissolved in 3 mL of DMSO. Molten nutrient agar (45 mL) was poured on sterile Petri plates and allowed to solidify. A bacterial lawn was spread on each plate by dispensing 7 mL of sterile soft agar containing 100 mL of cultures of the test organisms. The wells were dug with a 6 mm sterile metallic borer at appropriate distances. Then, 100 μL of the sample was poured into each well, and the plates were incubated at 37 °C for 24 h. The results in terms of inhibition zones were noted. Imipenem, a broad-spectrum β-lactam antibiotic, was used as a positive control, and DMSO as a negative control.

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