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Metabolites of the Fungistatic Agent 2β-Methoxyclovan-9α-ol by *Macrophomina phaseolina*

Syed Ghulam Musharraf,[*,†] Asma Najeeb,[†] Rahat Azher Ali,[†] Abida Amir Ali,[†] and Muhammad Iqbal Choudhary[†,§]

[†]HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

[§]Department of Chemistry, College of Science, King Saud University, Riyadh 1145, Saudi Arabia

ABSTRACT: Biotransformation of 2β-methoxyclovan-9α-ol (**1**), a fungistatic agent against *Botrytis cinerea*, was investigated with *Macrophomina phaseolina*. Demethoxylation, regioselective oxidation at C-9 and C-13, and inversion of the configuration at C-9 of compound **1** afforded six oxidative metabolites, 2β-methoxyclovan-9-one (**2**), clovan-2β,9β-diol (**3**), clovan-2β,9α-diol (**4**), clovan-2β,13-diol-9-one (**5**), 2β-methoxyclovan-9α,13-diol (**6**), and clovan-2β,9β,13-triol (**7**). Compounds **5**−**7** are described here for the first time, and their structures were deduced by different spectroscopic techniques. The antifungal activity of new metabolites **5**−**7** was also evaluated against *B. cinerea*.

KEYWORDS: biocatalysis, *Macrophomina phaseolina*, 2β-methoxyclovan-9α-ol, antifungal activity

INTRODUCTION

Bioconversions of terpenoids have a particular significance due to their uses as fragrances and flavoring agents and for their antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, and anthelmintic properties.[1−3] In continuation of our studies on biotransformational bioactive terpenes,[4−6] we describe here the biotransformational studies of compound **1** with *Macrophomina phaseolina*.

Compound **1**, a cyclized and rearranged product of (−)-caryophyllene oxide, showed a potent antifungal activity (83% at 200 ppm) against the plant pathogenic fungus *Botrytis cinerea*,[7] the microorganism that causes serious losses of many commercial crops including grapes, carrots, tomatoes, lettuce, tobacco, and strawberries.[8,9] Moreover, the rapid development of tolerance to commercially available fungicides by *B. cinerea* has led to an increasing use of fungicidal compounds. Considerable attention has been paid by Collado et al. to the rational design of fungicides against *B. cinerea* by the microbial transformation of botrydial and its structurally related compounds.[10] Similarly, microbial transformation of compound **1** was also studied by Collado et al. with *B. cinerea*. However, their transformed products were found to be less active in comparison to compound **1**.[7] We have incubated compound **1** with *M. phaseolina*, which afforded six polar compounds **2**−**7**. The fungistatic activity of biotransformed products **2**−**7** was also evaluated against *B. cinerea*.

EXPERIMENTAL PROCEDURES

General Experimental Conditions. An FTIR-8900 spectrophotometer was used to record IR spectra in $CHCl_3$. The melting points were determined on a Buchi 535 melting point apparatus and were uncorrected. A JASCO DIP-360 digital polarimeter was used to measure optical rotations in chloroform. The ^{1}H NMR spectra were recorded at 500 MHz, whereas ^{13}C NMR spectra were recorded on a Bruker AMX-500 operating at 125 MHz using $CDCl_3$ as solvent. Chemical shifts were reported in δ (ppm), relative to $SiMe_4$ as internal standard, and coupling constants (J) were measured in hertz. The electron impact (EI) and high-resolution fast atomic bombardment mass spectrometric (HRFAB MS) analyses were measured on a JEOL HX 110 mass spectrometer. Compound **1** was synthesized according to the protocol of Collado et al.[11] and purified by column chromatography (CC) with isocratic system of ethyl acetate (AcOEt)/petroleum ether (15:85), which afforded **1** with 96% purity. Qualitative analysis and purity of the compounds were checked by thin layer chromatography (TLC) (Si gel precoated plates, PF_{254}, 20 × 20, 0.25 mm, Merck) under UV light at 254 nm or by heating after spraying with ceric sulfate. All reagents used were of analytical grade.

General Fermentation and Extraction Conditions. Stock cultures were maintained at 4 °C on agar slants (Sabouraud dextrose agar). The medium for *M. phaseolina* (KUCC 730) was prepared by mixing the following ingredients into distilled H_2O (3.0 L): glucose (30.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), NaCl (15.0 g), and glycerol (30 mL). The fermentation medium thus obtained was adjusted to pH 7.0, distributed among 30 flasks of 250 mL capacity (100 mL in each), and then autoclaved at 121 °C for 20 min. Compound **1** (600 mg) dissolved in 15 mL of acetone was evenly distributed among 30 flasks (20 mg/0.5 mL in each flask), containing 24-h-old stage II cultures, and fermentation was continued for further 6 days on a rotatory shaker (200 rpm) at 29 °C. During the fermentation period, aliquots from culture were taken out daily and analyzed by TLC to determine the degree of transformation of substrate. In all experiments, one control flask, without fungus (to check substrate stability) and another flask without 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

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Table 1. ^{1}H and ^{13}C NMR Chemical Shift Data for Compounds 1 and 5−7 in $CDCl_3$

	1		5		6		7	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		44.2		44.8		46.2		46.2
2	3.31 t (5.7)	90.1	3.57 t (6.5)	79.6	3.19 t (5.8)	90.1	3.69 t (6.4)	79.4
3	1.74 m, 1.45 m	44.0	2.05 m, 1.81 m	44.2	2.41 m, 1.72 m	39.5	1.82 m, 1.62 m	43.1
4		36.7		47.4		44.5		44.5
5	1.46 m	50.4	2.17 m	41.8	1.81 m	43.6	1.92 m	43.0
6	1.31 m, 1.25 m	20.4	0.97 m, 1.71 m	21.0	1.37 m, 1.22 m	21.5	1.01 m, 1.32 m	21.0
7	1.15 m, 1.39 m	33.2	1.92 m, 1.13 m	31.3	1.18 m, 1.43 m	33.6	1.75 m, 1.42 m	30.9
8		34.7		45.7		30.2		34.8
9	3.32 t (4.3)	75.2		217.2	3.42 t (4.2)	74.7	3.13 dd (5.4, 11.2)	77.5
10	1.51 m, 1.49 m	25.8	2.41 m, 1.73 m	38.2	1.56 m, 1.48 m	26.1	1.78 m, 1.59 m	27.6
11	1.21 m, 1.14 m	26.3	1.62 m, 1.51 m	35.7	2.12 m, 1.62 m	27.6	1.68 m, 1.51 m	28.4
12	1.33 m, 0.97 m	36.4	1.42 m, 1.55 m	41.1	1.52 m, 0.99 m	35.9	1.24 m, 0.98 m	41.9
13	1.03 s	31.1	3.24 d (9.6)	70.6	3.29 d (9.8)	72.2	3.29−3.30[a] (2H, m)	70.6
			3.34 d (9.6)		3.31 d (9.8)			
14	0.86 s	25.2	1.05 s	25.5	0.98 s	21.5	0.94 s	21.4
15	0.97 s	28.2	1.01 s	23.1	0.96 s	28.4	0.96 s	29.1
OCH_3	3.33 s	57.9			3.31 s	57.8		

[a] Overlapping, ^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz.

harvested and compared with the test by TLC to confirm the presence of biotransformed products. After filtration, extraction, and evaporation, brown gum (2.11 g) was obtained, which after repeated CC with gradient system of petroleum ether and ethyl acetate (AcOEt) yielded **2** (7.4 mg; 1.2% yield; 97% purity; with petroleum ether/AcOEt 74:26), **3** (10.5 mg; 1.7% yield; 95% purity; with petroleum ether/AcOEt 65:35), **4** (11.4 mg; 1.9% yield; 94% purity; with petroleum ether/AcOEt 64:36), **5** (8.8 mg; 1.4% yield; 96% purity; with petroleum ether/AcOEt 60:40), **6** (18.7 mg; 3.1% yield; 97% purity; with petroleum ether/EtOAc 55:45), and **7** (28.8 mg; 4.8% yield; 97% purity; with petroleum ether/EtOAc 50:50).

Clovan-2*β*,13-diol-9-one (5): white crystalline solid; $C_{15}H_{24}O_3$; mp 101−102 °C; $[\alpha]^{25}_D$ = +8.7 (*c* 0.01, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 3436, 2938, 2870, 1735; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.57 (t, *J* = 6.5 Hz, H-2α), 3.24 and 3.34 (d, *J* = 9.6 Hz, H_2-13); ^{13}C NMR (125 MHz, $CDCl_3$) δ 79.6 (C-2), 70.6 (C-13), 217.6 (C-9); for detailed ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; EI-MS 234 (5, $[M^+ - H_2O]$), 210 (7, $[M - CH_2OH]^+$) 185 (32), 172 (38), 159 (32), 105 (41), 99 (100); HRFAB-MS 253.1839 ($[M + H]^+$, $C_{15}H_{25}O_3$; calcd 253.1804).

2*β*-Methoxyclovan-9α,13-diol (6): colorless crystalline solid; $C_{16}H_{28}O_3$; mp 119−120 °C; $[\alpha]^{25}_D$ = +21.7 (*c* 0.08, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 3354, 2941, 2868, 1124; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.19 (t, *J* = 5.8 Hz, H-2α), 3.42 (t, *J* = 4.2 Hz, H-9*β*), 3.29 and 3.31 (d, *J* = 9.8 Hz, H_2-13), 3.31 (s, OMe); ^{13}C NMR (125 MHz, $CDCl_3$) δ 90.1 (C-2), 74.7 (C-9), 72.2 (C-13), 57.8 (OMe); for detailed ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; EI-MS 250 (11, $[M^+ - H_2O]$), 218 (48, $[M - H_2O - MeOH]^+$), 200 (37, $[M - 2H_2O - MeOH]^+$), 187 (46), 174 (63), 161 (78), 145 (22), 107 (52), 99 (100).; HRFAB-MS 269.2135 ($[M + H]^+$, $C_{16}H_{29}O_3$; calcd 269.2117).

Clovan-2*β*,9*β*,13-triol (7): white crystalline solid; $C_{15}H_{26}O_3$; mp 151−152 °C; $[\alpha]^{25}_D$ = +17.4 (*c* 0.05, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 3329, 2931, 2877 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.69 (t, *J* = 6.4 Hz, H-2α), 3.13 (dd, *J* = 5.4, 11.2 Hz, H-9α), 3.29−3.30 (m, overlapped, H_2-13); ^{13}C NMR (125 MHz, $CDCl_3$) δ 79.4 (C-2), 77.5 (C-9), 70.6 (C-13); for detailed ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; EI-MS 236 (8, $[M - H_2O]^+$), 222 (7, $[M - CH_2OH]^+$), 218 (12, $[M - 2H_2O]^+$4), 181 (82), 187 (42), 147 (53), 105 (56), 93 (68), 71 (100); HRFAB-MS 255.1955 ($[M + H]^+$, $C_{15}H_{27}O_3$; calcd 255.1960).

Antifungal Assay. *B. cinerea* (KUCC 869) used in this work was isolated from the infected strawberries, identified by the Mycological Department of Botany, University of Karachi. Miconazole was used as a standard drug. An agar tube dilution method[12] was used for the screening of antifungal activity of pure compounds. Stock solutions of pure compounds (1 mg) were prepared in sterile DMSO (1 mL). Test compounds were added from the stock solution to nonsolidified Sabouraud agar media (SDA). The final concentration of pure compounds was made as 200 μg/mL. Tubes were allowed to solidify at room temperature in slanting position and inoculated with 4 mm diameter of inocula, derived from 7-days-old fungal culture. These tubes were then incubated at 27−29 °C for 7 days. The growth in the compounds containing media was measured by linear growth (mm), and percent growth inhibition was calculated with reference to the respective control.

RESULTS AND DISCUSSION

Compound **1** was obtained from (−)-caryophyllene oxide by the cyclization and rearrangement with tetracyanoethylene (TCNE) in methanol.[11] *M. phaseolina* for the biotransformation of compound **1** was selected on a preparative scale after a screening experiment. Six days of incubation of compound **1** with *M. phaseolina* afforded six compounds, **2**−**7**. Structures of these compounds were elucidated through comparative spectral studies with compound **1**. Metabolites **2** (2*β*-methoxyclovan-9-one), **3** (clovan-2*β*,9α-diol), and **4** (clovan-2*β*,9*β*-diol) were identified as the known compounds through comparison of their spectroscopic data.[7,10]

Compound **5** was isolated as a white crystalline compound and identified as a clovan-2*β*,13-diol-9-one on the basis of the following spectroscopic evidence. The HRFAB MS of **5** showed the $[M + H]^+$ at *m*/*z* 253.1839, corresponding to the formula $C_{15}H_{25}O_3$ (calcd 253.1804). The IR spectrum showed an additional absorption at 1735 cm^{-1}, indicating the presence of carbonyl functionality in comparison of **1**. The ^{1}H NMR

Table 2. Antifungal Activity Results of Compounds 1−7 against *Botrytis cinerea*

compd	% inhibition[a]	compd	% inhibition[a]
1	81	**5**	8
2	71	**6**	17
3	59	**7**	11
4	32		

[a] Concentration = 200 μg/mL.

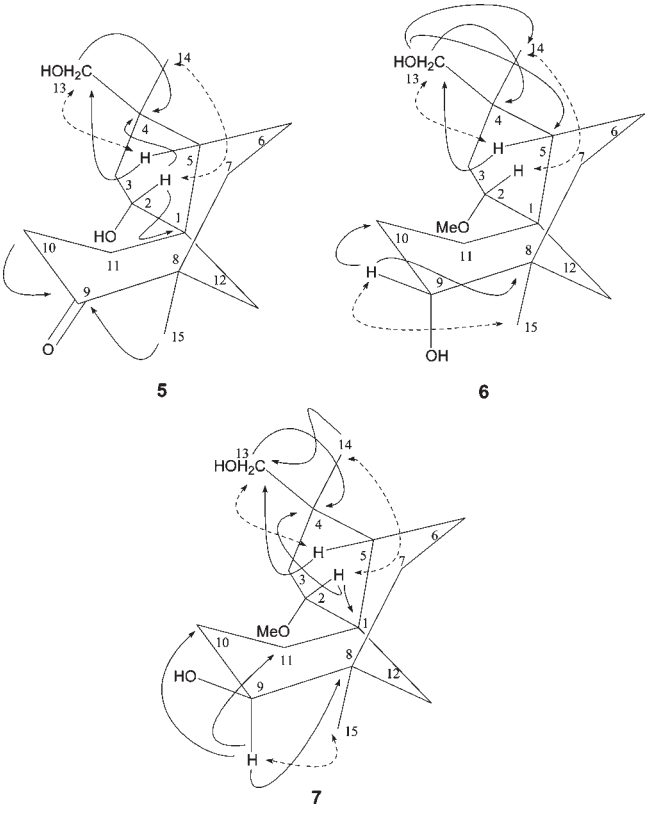


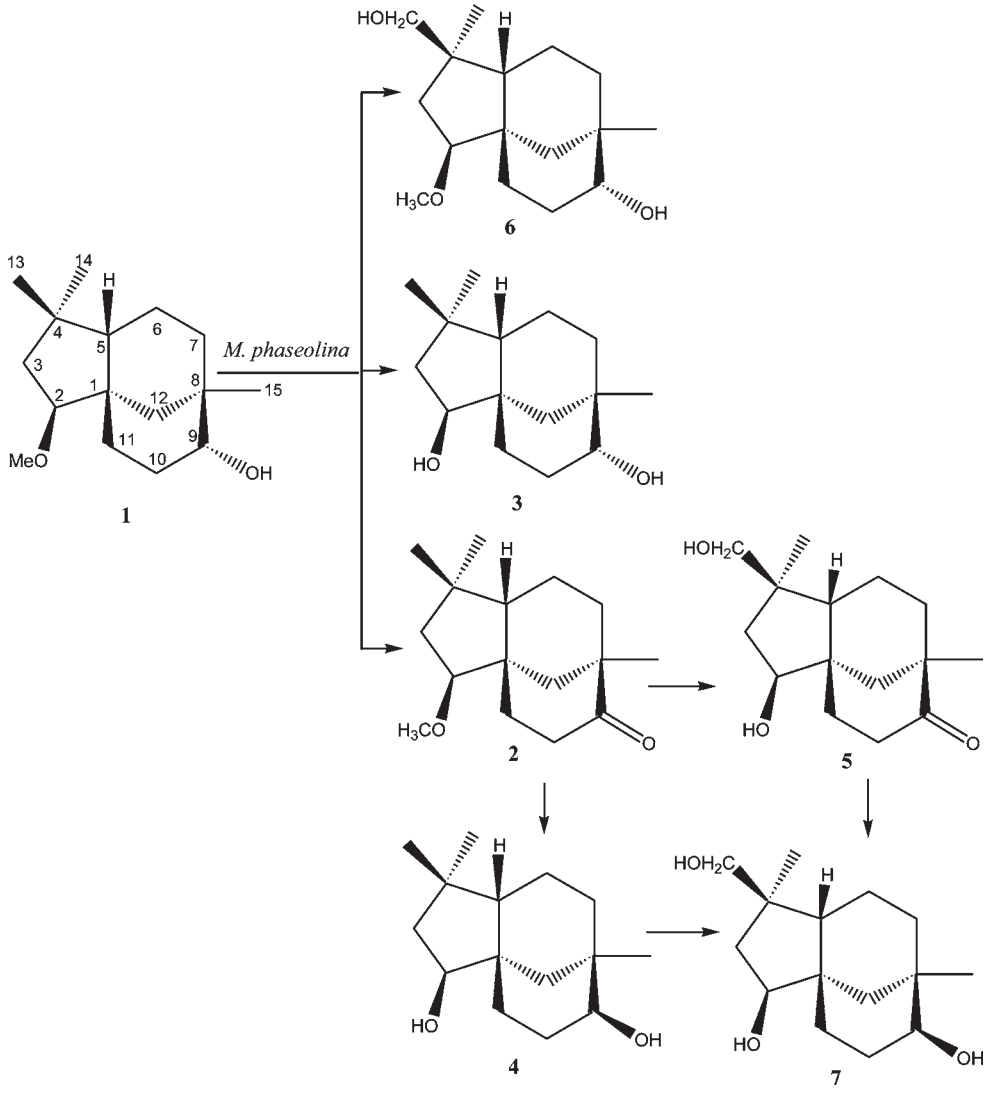
Figure 1. Key NOESY (dashed arrows) and HMBC (solid arrows) interactions in new compounds **5**−**7**.

spectrum (Table 1, $CDCl_3$) of compound **5** showed close resemblance with compound **1**. An additional AB doublet appeared at δ 3.24 (J = 9.6 Hz) and 3.34 (J = 9.6 Hz), corresponding to CH_2OH, whereas H-9 and C-2 oxymethyl signals disappeared and H-2 showed a downfield shift at δ 3.57. The ^{13}C NMR (Table 1) spectrum showed the resonances for 15 carbon signals including 2 methine, 7 methylene, 2 methyl, and 4 quaternary carbons. Two additional signals appeared at δ 70.6 and 217.2, corresponding to C-13 and C-9, respectively. An upfield shift at C-2 was observed at δ 79.6 due to demethoxylation. Similarly, a downfield shift of C-10 (δ 38.2) and C-8 (δ 45.7) and an upfield shift of C-5 (δ 41.8) further supported the carbonylation at C-9 and hydroxylation at one of the C-4 *gem*-methyl groups, respectively. The position and stereochemistry of newly introduced hydroxyl groups were further deduced by 2D NMR techniques. The COSY 45° spectrum showed couplings between H-2 (δ 3.57) and H_2-3 (δ 2.05, 1.81), whereas the heteronuclear multiple bond correlation (HMBC) spectrum showed interactions between H-2 (δ 3.57) and C-1 (δ 44.8) and C-4 (δ 47.4), between H_3-15 (δ 1.01) or H_2-10 (δ 2.41, 1.73) and C-9 (δ 217.0), and between H_2-13 (δ 3.34, 3.24) and C-4 (δ 47.4). The nuclear Overhauser enhancement spectroscopy (NOESY) spectrum of compound **5** (Figure 1) showed interactions between H_2-13 and βH-5, indicating the hydroxylation of the β-orientated C-4 *gem*-methyl group, which further supported the structure as clovan-2β,13-diol-9-one (**5**).

Compound **6** was obtained as a colorless crystalline solid and characterized as 2β-methoxyclovan-9α,13-diol. The HRFAB-MS showed a $[M + H]^+$ peak at m/z 269.2135 corresponding to the formula $C_{16}H_{29}O_3$ (calcd 269.2117). EI-MS showed significant fragment ions at m/z 250 and 218, corresponding to $[M^+ - H_2O]$ and $[M^+ - H_2O + MeOH]$, respectively. The IR spectrum of **6** showed hydroxyl absorption at 3371 cm^{-1}. The ^{1}H NMR spectrum of compound **6** was similar to that of **1** with an additional AB doublet of CH_2OH at δ 3.31 (J = 9.8 Hz) and 3.29 (J = 9.8 Hz) and the disappearance of the Me-13 signal. The ^{13}C NMR included broad-band decoupled and distortionless enhancement by polarization transfer (DEPT) spectra of compound **6** and showed the disappearance of the C-13 methyl carbon and the appearance of an additional hydroxyl-bearing methylene signal at δ 72.2, when compared with compound **1** (Table 1). Moreover, γ-upfield shift of C-3 (δ 39.5) and C-5 (δ 43.6) further supported the hydroxylation at one of the C-4 *gem*-dimethyl groups. The C-13 protons (δ 3.31, 3.29) also showed HMBC interactions with C-5 (δ 43.6), C-4 (δ 44.5), and C-14 (δ 21.5). NOESY correlations between H_2-13 and H-5β supported the β-orientation of the C-13 hydroxymethylene group (Figure 1). These observations supported the structure of the metabolite as 2β-methoxyclovan-9α,13-diol (**6**).

Compound **7** was isolated as a white crystalline solid and exhibited a $[M + H]^+$ peak at m/z 255.1955, corresponding to the formula $C_{15}H_{27}O_3$ (calcd 255.1960) in the HRFAB-MS of **7**. The IR spectrum of **7** showed hydroxyl absorption at 3385 cm^{-1}. The ^{1}H NMR spectrum of compound **7** exhibited additional multiplets at δ 3.29−3.30 corresponding to CH_2OH and the disappearance of the Me-13 signal. A downfield shift of the H-2 (δ 3.69) signal and the absence of the C-2 methoxy signal indicated the demethoxylation. A remarkable feature was the change of coupling constant and multiplicity of H-9 signal (δ 3.13, dd, $J_{9ax-10ax}$ = 11.2 Hz, $J_{9ax-10eq}$ = 5.4 Hz) geminal to a OH, indicating the β (equatorial) orientation of the C-9 OH group in comparison to **1**. C-9 inversion is a reported phenomenon in compound **1** by *B. cinerea* via asymmetric reduction of ketonic intermediate.[7] Most likely, compound **7** is formed from the steroselective reduction of C-9 keto group in **5** or via C-13 hydroxylation of **4** (Scheme 1). The ^{13}C NMR (broad-band decoupled and DEPT) spectra of compound **7** showed the disappearance of the C-13-Me and OMe carbon signals and the appearance of an additional hydroxyl-bearing methylene carbon signal at δ 70.6, whereas C-2 (δ 79.4) and C-9 (δ 77.5) showed an upfield and a downfield shift, respectively, when compared with the compound **1** (Table 1). Similarly, upfield shifts of C-3 (δ 43.1) and C-5 (δ 43.0) further supported the hydroxylation at one of the C-4 *gem*-methyl groups. The COSY 45° spectrum of compound **7** showed cross peaks H-2 (δ 3.69)/H_2-3 (δ 1.82, 1.62) and H-9 (δ 3.13)/H_2-10 (δ 1.78, 1.59). The C-9 proton (δ 3.13) also showed HMBC with C-8 (δ 34.8), C-10 (δ 27.6), and C-11 (28.4), whereas H-2 (δ 3.69) showed correlations with C-1 (δ 46.2) and C-4 (δ 44.5). The H_3-14 showed HMBC interactions with C-13 (δ 70.6). The orientations of hydroxyl

Scheme 1. Microbial Transformation of Compound 1 by *Macrophomina phaseolina*



groups at C-9 and C-2, as well as of the hydroxymethyl at C-4, were established by qualitative and quantitative NOE experiments. The NOESY experiment showed interactions between H_2-13 and H-5β, between H-2 and H_3-14, and between H-9 and H_3-15 (Figure 1). Moreover, a nuclear Overhauser enhancement differential (NOED) experiment showed the enhancement of H-9β (2.96%) and H-2α (2.83%) signals by the irradiation of H_3-15 (δ_H 0.96) and H_3-14 (δ_H 0.94), respectively, which further supported the structure as clovan-2β,9β,13-triol (**7**).

M. phaseolina showed a very similar metabolic pathway of compound **1** as reported with *B. cinerea*;[7] metabolites **2**−**4** were the common transformed products, whereas dihydroxy derivatives (**5**−**7**) of **1** were exclusively formed by *M. phaseolina*. Compounds **1**−**7** were evaluated for their fungistatic activity against *B. cinerea*. Miconazole was used as a positive control in the antifungal assay. Transformed products **2**−**7** were found to be weaker than **1** against *B. cinerea* (Table 2). The results showed that the incorporation of oxygen functionalities led to the decrease in antifungal activity of parent compound **1** against *B. cinerea*. Moreover, Collado et al.[13] has recently investigated the structure−antifungal activity relationship of various derivatives of clovane against *B. cinerea* and found compounds possessing a nitrogen atom in the side chain attached at C-2 to have potent activity in comparison with **1**. Compounds **5** and **7** can serve as new precursors for the similar derivative preparation and may have better antifungal profiles against *B. cinerea*.

In conclusion, these biotransformed products are of interest for several reasons. Regioselective hydroxylation at the C-14 methyl group, oxidation of the C-9 hydroxyl group into a keto group, inversion of the configuration C-9α to C-9β-OH, and facile dealkylation of the 2β-methyl ether to generate 2β,9α-diols were observed. These transformations provide access to the clovane derivatives, which were difficult to produce by chemical methods. However, the antifungal activity of the biotransformed products was not promising as compared to substrate **1**.

AUTHOR INFORMATION

Corresponding Author

*Phone: +92 213 4824924-5 or +92 213 4819010. Fax: + 92 21 4819018-9. E-mail: musharraf1977@yahoo.com.

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