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# Microbial transformation of 5α-hydroxycaryophylla-4(12), 8(13)-diene with *Macrophomina phaseolina*

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ABSTRACT

Biotransformation of 5α-hydroxycaryophylla-4(12), 8(13)-diene (**1**) by the fungus *Macrophomina phaseolina* resulted into regioselective oxidation at C-4(12) exocyclic double bond and C-11 *gem*-methyl groups, leading to the formation of three new metabolites, 4β-methoxycaryophyllene-5α, (11*S*)-14-diol (**2**), 4β-methoxycaryophyllene-5α, (11*R*)-15-diol (**3**), and caryophyllene-5α, (11*R*)-15-diol (**4**). The structures of metabolites were deduced by spectroscopic and single-crystal X-ray diffraction analysis. Compounds **1**–**4** were evaluated for *in vitro* antimalarial activity, where compound **2** exhibited an $IC_{50}$ value of $0.72 \pm 0.17$ μg/ml (standard chloroquine diphosphate, $IC_{50} = 0.025 \pm 0.01$ μg/ml).



## 1. Introduction

Many terpenoids have particular significance as fragrances or flavoring agents and also possess antibacterial, antiviral, or cytotoxic activities [1–3]. We have recently investigated the biotransformation of various bioactive terpenes with fungal or plant cell cultures [4–6]. This paper describes the biotransformation of a sesquiterpene **1** with *Macrophomina phaseolina*.

5α-Hydroxycaryophylla-4(12), 8(13)-diene (**1**), a caryophyllene-type sesquiterpene, is a constituent of many essential oils of folk medicinal plants and spices [7–14]. Compound **1** was synthesized from the alcoholysis of caryophyllene oxide [15]. Microbial transformation of caryophyllene oxide derivatives has been reported earlier [16,17], but the biotransformation of 5α-hydroxycaryophylla-4(12), 8(13)-diene (**1**) is performed for the first time. Cell suspension culture of *M. phaseolina* has metabolized compound **1** into three polar compounds. These metabolites were unambiguously identified as 4β-methoxycaryophyllene-5α, (11*S*)-14-diol (**2**), 4β-methoxycaryophyllene-5α, (11*R*)-15-diol (**3**), and caryophyllene-5α, (11*R*)-15-diol (**4**) by spectroscopic and X-ray diffraction analysis. Moreover, compounds **1**–**4** were also screened against *Plasmodium falciparum* (3D7 strain) and chloroquine diphosphate was used as a standard for comparison.

## 2. Experimental

### 2.1. General experimental method

IR spectra were recorded in $CHCl_3$ on FTIR-8900 spectrophotometer. The melting points were determined on Buchi 535 melting point apparatus and were uncorrected. Optical rotations were measured on JASCO DIP-360 digital polarimeter in methanol. The $^1H$ NMR spectra were recorded on 300 MHz, while $^{13}C$ NMR spectra were recorded on Bruker AMX-500 operating at 125 MHz using $CDCl_3$ as solvent. Chemical shifts were reported in $\delta$ (ppm), relative to $SiMe_4$ as internal standard, coupling constants ($J$) were measured in Hz. The EI-MS and HREI-MS were measured on Jeol HX 110 mass spectrometer. TLC was performed with Si gel (Merck) precoated plates ($PF_{254}$, $20 \times 20$, 0.25 mm). Compound **1** was synthesized according to the protocol of Collado et al. [16]. Ceric sulphate spraying reagent was used for the staining of compounds on TLC. All reagents used were of analytical grades.

### 2.2. Fermentation of compound *1* with M. phaseolina

Stock cultures of *M. phaseolina* (KUCC 730) were maintained at 4 °C on agar slants (Sabouraud dextrose agar). The medium for *M. phaseolina* was prepared by mixing the following ingredients into distilled $H_2O$ (3.0 l): glucose (30.0 g), peptone (15.0 g), yeast

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**Table 1**
$^1$H NMR and $^{13}$C NMR chemical shift data for compounds **2**–**4** in $CDCl_3$.

| Position | **1** | | **2** | | **3** | | **4** | |
|---|---|---|---|---|---|---|---|---|
| | $\delta$H ($J$ in Hz) | $\delta$C | $\delta_H$ ($J$ in Hz) | $\delta_C$ | $\delta_H$ ($J$ in Hz) | $\delta_C$ | $\delta_H$ ($J$ in Hz) | $\delta_C$ |
| 1 | 1.67 *m*[a] | 54.3 | 1.95 *m* | 54.5 | 2.01 *m* | 49.2 | 1.96 *m* | 49.4 |
| 2 | 1.51–1.92 *m*[a] | 37.2 | 1.65–1.81 *m*[a] | 31.7 | 1.83 *m*, 1.48 *m* | 30.8 | 1.89 *m*, 1.62 *m* | 38.8 |
| 3 | 1.51–1.92 *m*[a] | 32.3[b] | 1.65–1.81 *m*[a] | 32.5[b] | 1.65–1.74 *m*[a] | 32.3[b] | 1.61–1.78 *m*[a] | 34.3[b] |
| 4 | – | 151.6 | – | 80.0 | – | 80.1 | – | 75.6 |
| 5 | 4.01 *dd* (3.7, 8.8) | 75.5 | 3.69 *t* (6.3) | 73.0 | 3.73 *t* (6.0) | 73.0 | 3.65 *t* (5.7) | 73.2 |
| 6 | 2.01 *m*, 2.25 *m* | 32.1[b] | 2.04 *m*, 2.44 *m* | 34.1 | 2.01 *m*, 2.45 *m* | 34.1 | 2.01 *m*, 2.42 *m* | 34.2[b] |
| 7 | 1.51–1.92 *m*[a] | 32.7[b] | 1.65–1.81 *m*[a] | 32.7[b] | 1.65–1.74 *m*[a] | 32.7[b] | 1.43 *m*, 1.87 *m* | 30.2 |
| 8 | – | 152.1 | – | 151.8 | – | 151.6 | – | 151.4 |
| 9 | 2.37 *m* | 43.2 | 2.42 *m* | 42.0 | 2.41 *m* | 41.6 | 2.38 *m* | 41.9 |
| 10 | 1.35 *m*, 1.68 *m* | 30.2 | 1.41 *m*, 1.72 *m* | 22.1 | 1.42, 1.71 *m* | 22.9 | 1.35 *m*, 1.65 *m* | 23.5 |
| 11 | – | 33.6 | – | 38.4 | – | 38.0 | – | 38.5 |
| 12 | 4.78 *s*, 4.77 *s* | 113.6 | 1.04 *s* | 15.6 | 1.05 *s* | 15.7 | 1.12 *s* | 21.9 |
| 13 | 4.94 *s*, 4.97 *s* | 110.8 | 4.91 *brs* | 110.7 | 4.91 *brs* | 110.8 | 4.93 *s*, 4.92 *s* | 110.7 |
| 14 | 0.98 *s* | 30.4 | 3.56–3.62 *m*[a] | 67.4 | 1.04 *s* | 17.6 | 1.02 *s* | 17.6 |
| 15 | 0.98 *s* | 22.1 | 1.08 *s* | 25.1 | 3.34 *brs* | 71.7 | 3.34 *brs* | 71.2 |
| $OCH_3$ | – | – | 3.16 *s* | 48.5 | 3.15 *s* | 48.5 | – | – |

$^1$H NMR at 300 MHz and $^{13}$C NMR at 125 MHz.
[a] Overlapping.
[b] Interchangeable assignment.

extract (15.0 g), $KH_2PO_4$ (15.0 g) and NaCl (15.0 g). The fermentation medium thus obtained was distributed among 30 flasks of 250 ml capacity (100 ml in each) and autoclaved at 121 °C for 20 min. The substrate (620 mg) was dissolved in acetone (15 ml) and the resulting clear solution was evenly distributed among 30 flasks (66.6 μl/0.5 ml in each flask), containing 24-h-old stage II cultures and fermentation was carried out for further 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 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 without exogenous substrate (for checking endogenous metabolite) 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 thin layer chromatography. Control flasks were also harvested and compared with the test by TLC to confirm the presence of biotransformed products. After filtration, extraction and evaporation, brown gum (0.97 g) was obtained which after repeated CC (petroleum ether/AcOEt gradient) yielded **1** (16.4 mg; with petroleum ether/AcOEt 91: 09), **2** (22.1 mg; with petroleum ether/AcOEt 74: 26), **3** (47.6 mg; with petroleum ether/AcOEt 72: 28) and **4** (17.1 mg; with petroleum ether/EtOAc 68: 32).

#### 2.2.1. 4β-Methoxycaryophyllene-5α, (11S)-14-diol (**2**)

Colorless liquid; $C_{16}H_{28}O_3$; $[\alpha]_D^{25} = -31.4$ ($c = 0.04$, $CHCl_3$); IR ($CHCl_3$) $\nu_{max}$ cm$^{-1}$: 3432, 2934, 2865, 1445, 1086, 1045; $^1$H NMR and $^{13}$C NMR spectroscopic data, see Table 1; EI-MS: 268 (7, $M^+$), 253 (3, $[M-Me]^+$), 236 (6, $[M-MeOH]^+$), 218 (9), 205 (11), 181 (14), 159 (18), 125 (17), 107 (34), 85 (100), 79 (48); HREI-MS: 268.2037 ($M^+$, $C_{16}H_{28}O_3$; calc. 268.2038).

#### 2.2.2. 4β-Methoxycaryophyllene-5α, (11R)-15-diol (**3**)

Colorless crystalline solid; $C_{16}H_{28}O_3$; M.P. 82–83 °C; $[\alpha]_D^{25} = -53.7$ ($c = 0.031$, $CHCl_3$); IR ($CHCl_3$) $\nu_{max}$ cm$^{-1}$: 3430, 2933, 2867, 1446, 1084, 1044; $^1$H NMR and $^{13}$C NMR spectroscopic data, see Table 1; EI-MS: 268 (7, $M^+$), 253 (3, $[M-Me]^+$), 236 (2, $[M-MeOH]^+$), 218 (5), 205 (18), 181 (21), 159 (17), 125 (28), 107 (15), 85 (100), 79 (40); HREI-MS: 268.2033 ($M^+$, $C_{16}H_{28}O_3$; calc. 268.2038).

#### 2.2.3. Caryophyllene-5α, (11R)-15-diol (**4**)

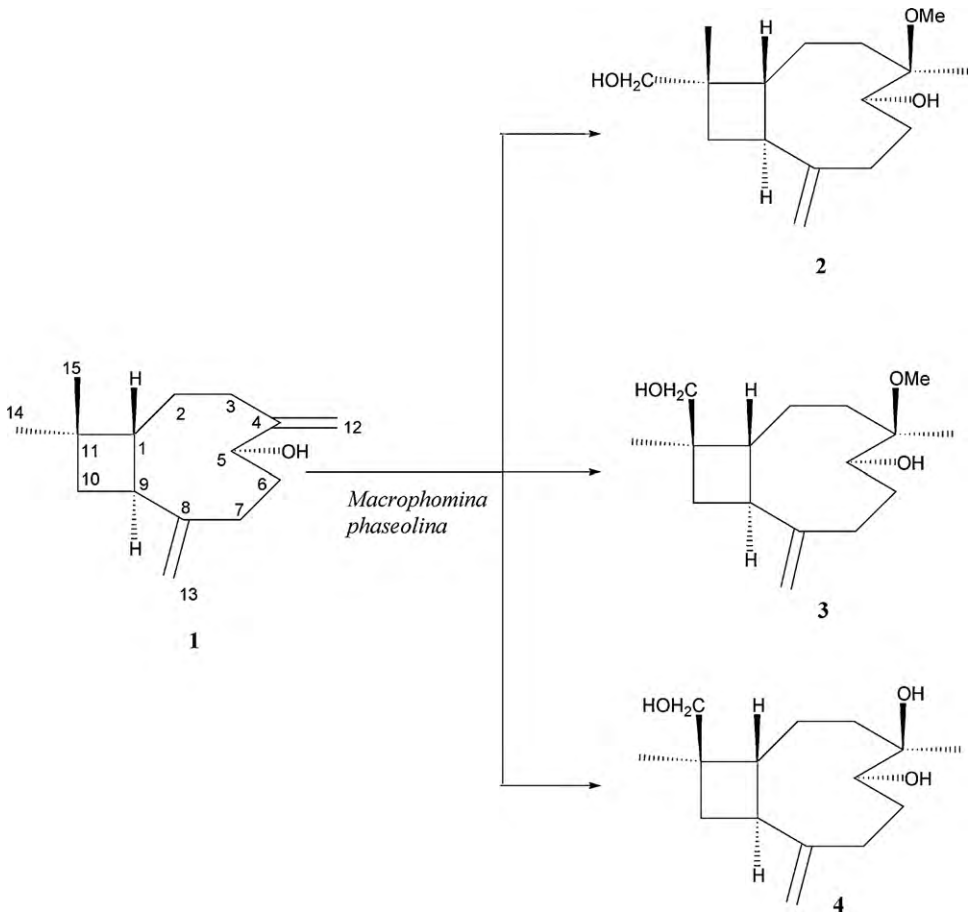
Colorless crystalline solid; $C_{15}H_{26}O_3$; M.P. 97–98 °C; $[\alpha]_D^{25} = -21.4$ ($c = 0.011$, $CHCl_3$); IR ($CHCl_3$) $\nu_{max}$ cm$^{-1}$: 3379, 2929, 2864, 1443, 1054; $^1$H NMR and $^{13}$C NMR spectroscopic data, see Table 1; EI-MS: 254 (3, $M^+$), 236 (6, $[M-H_2O]^+$), 223 (20), 205 (12), 179 (24), 155 (44), 125 (84), 107 (39), 93 (100); HREI-MS: $m/z$ 254.1857 ($M^+$, $C_{15}H_{26}O_3$; calc. 254.1882).

### 2.3. Crystallographic data of compound **3**

The structure of compound **3** was unambiguously determined by single-crystal X-ray diffraction technique. A suitable crystal of compound **3** was obtained by recrystallization from petroleum ether–$CH_2Cl_2$–acetone (2:2:1). A colorless crystal with dimensions 0.20 mm × 0.14 mm × 0.10 mm was selected for the crystallographic measurements. $C_{16}H_{28}O_3$: $M_r$ 268.38; tetragonal, $\boldsymbol{a} = 11.1970$ (10), $\boldsymbol{b} = 11.1970$ (10), $\boldsymbol{c} = 24.795$ (4) Å, $\alpha = \beta = \gamma = 90.0°$, $V = 3108.6$ (6) Å$^3$, space group = $P4_12_12$, $Z = 8$, $D_{calc} = 1.147$ Mg/m$^3$, $F(000) = 1184$, Mo-Kλ = 0.71073 Å. Unit cell dimensions were determined by least squares fit of 1715 reflections, measured at 293° (2) K using Mo-Kα radiations on a Nonius KappaCCD diffractometer. The intensity data within ($\theta$) range of 3.06–27.47° were collected at 173° (2) K. A total of 5935 reflections were recorded, of which 3559 reflections were judged on the basis of $I > 2\sigma(I)$. The structure was solved by the direct methods [18] and expanded using Fourier transformation techniques [19]. The structure was refined by a full-matrix least-square calculation on $F^2$ with the aid of program [20]. The final $R$ and $R_w$ factors were measured as 0.0369 and 0.0892, respectively. The figures were plotted with the aid of ORTEPII program [21]. Crystallographic data for compound **3** have been deposited in Cambridge Crystallographic Data Center, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44 1223 336 033; e-mail: deposit@ccdc.cam.ac.uk).

### 2.4. Parasite lactate dehydrogenase assay for antimalarial activity

*In vitro* antimalarial activity was performed by Parasite Lactate Dehydrogenase Assay (pfLDH) [22] using *P. falciparum* 3D7 strain. Pure compounds and standard drugs were serially diluted (twofold) with complete culture medium in 96-well plates. Infected RBCs solution with 2% parasitemia and 1% hematocrit was added, making the total volume 200 μl in each well. Plates were incubated in




**Scheme 1.** Metabolism of compound **1** by *Macrophomina phaseolina*.

candle jar with 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 37 °C for 72 h. For malstat reaction, in 100 μl of malstat solution, 20 μl solution from each well of Plate 1 to respective well of malstat plate was added. Plates were placed in shaking water bath at 37 °C for 30 min. 25 μl solution (1:1 solution of NBT (nitro blue tetrazolium), 2 mg/ml) and PES (phenazine ethano sulphate, 0.1 mg/ml) was added in each well and the plates were kept in the dark for complete reaction. Plates were read at 650 nm and the OD was recorded. $IC_{50}$ was calculated with the help of EZfit (Software for enzyme inhibition kinetic study by Perrella Scientific) computer program.

## 3. Results and discussion

5α-Hydroxycaryophylla-4(12), 8(13)-diene (**1**) is obtained from (−)-caryophyllene oxide by reacting with tetracyanoethylene (TCNE) in methanol [16]. Screening scale experiment showed that *M. phaseolina* has the ability to transform compound **1** into polar metabolites. Preparative scale fermentation was thus carried out to produce sufficient quantities of metabolites for detailed structural studies and bioactivity evaluation. 6-Day incubation of compound **1** with *M. phaseolina* afforded three new compounds **2**–**4** (Scheme 1). Structures of these compounds were elucidated through comparative spectral studies with sample **1**.

Compound **2** was isolated as a colorless liquid. The EI-MS of compound **2** displayed the $M^+$ peak at *m/z* 268, while other significant ions were at *m/z* 253 $[M-Me]^+$ and 236 $[M-MeOH]^+$. The HREI-MS showed the $M^+$ at *m/z* 268.2037, corresponding to the molecular formula $C_{16}H_{28}O_3$ (calc. 268.2038). IR absorption at 3432 $cm^{-1}$ of **2** indicated the presence of a hydroxyl group. $^1H$ NMR spectrum of **2** was found to be substantially different from **1** in many aspects. First the disappearance of C-4/12 olefinic signals, secondly the appearance of two additional signals; oxy-methylene protons at $\delta_H$ 3.56–3.62 (*m*) and methoxy protons at $\delta_H$ 3.16 (*s*). Similarly, $^{13}C$ NMR spectrum of **2** showed two additional signals at $\delta_C$ 67.4 and 48.5, corresponding to oxy-methylene and methoxy carbons, respectively. Moreover, up-field shift of C-4 quaternary carbon at δ 80.0 and γ-up-field shift of C-10 ($\delta_C$ 22.1), in comparison of **1** further supported the Markonikov methoxylation of C-4/12 exomethylene double bond and hydroxylation at one of the C-11 *gem*-methyl groups (Table 1). HMBC spectra showed correlations of $H_3$-12 ($\delta_H$ 1.04), H-5 ($\delta_H$ 3.69), and MeO ($\delta_H$ 3.16) with C-4 ($\delta_C$ 80.0); $H_3$-12 ($\delta_H$ 1.04) and $H_2$-6 ($\delta_H$ 2.44, 2.04) with C-5 ($\delta_C$ 73.0); $H_3$-15 ($\delta_H$ 1.08), H-1 ($\delta_H$ 1.95), and $H_2$-10 ($\delta_H$ 1.72, 1.41) with C-14 (δ 67.4). This further supported the structure of metabolite as **2**. The stereochemistry of newly formed chiral centers, C-4 and C-11, was established as *R* and *S*, respectively, by qualitative and quantitative NOE experiments. NOESY experiment showed correlations between H-9α/$H_2$-14 and $H_3$-12α, H-5β/H-1β and OMe (Fig. 1), indicating that $H_2$-14 and OMe were in α and β orientations, respectively. Moreover, NOED experiment showed the enhancement of H-5β (7.72%) and $H_3$-15β (1.64%) signals by the irradiation of H-1β ($\delta_H$ 1.95). Thus the structure of compound **2** was deduced as 4β-methoxycaryophyllene-5α, (11*S*)-14-diol.

Compound **3** was obtained as a colorless crystalline compound and found to be an epimer of compound **2**. The HREI-MS showed the $M^+$ at *m/z* 268.2033, in agreement with the formula $C_{16}H_{28}O_3$ (calc. 268.2038). The IR spectrum of compound **3** showed absorption at 3430 $cm^{-1}$, indicating the presence of hydroxyl group. $^1H$ NMR and $^{13}C$ NMR spectra of compound **3** showed the absences of C-4(12) exocyclic double bond and appearance of two additional signals

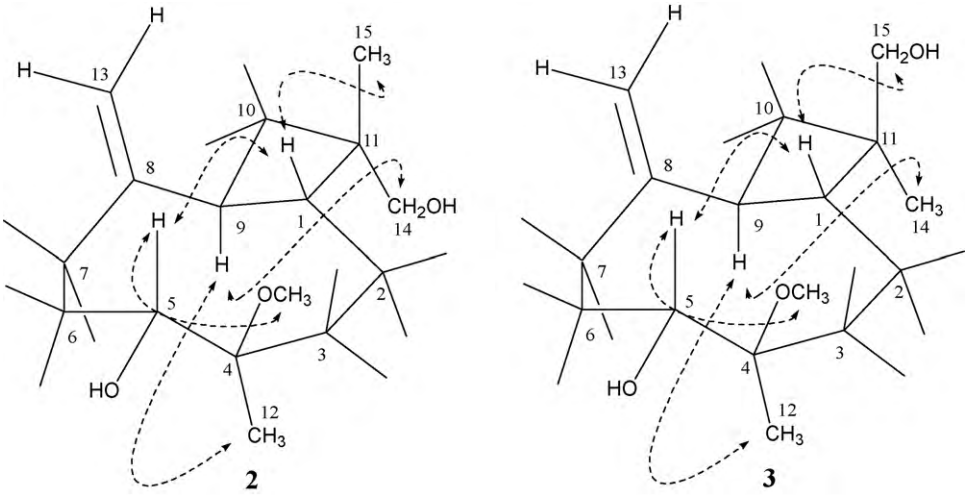



**Fig. 1.** Key NOESY correlations in compounds **2** and **3**.

for $CH_2OH$ ($\delta_H$ 3.34, *brs*; $\delta_C$ 71.7) and OMe ($\delta_H$ 3.15, *s*; $\delta_C$ 48.5), including up-field shift of C-4 ($\delta_C$ 80.1), C-1 ($\delta_C$ 49.2) and C-10 ($\delta_C$ 22.9) signals in comparisons of **1** (Table 1). This further supported the methoxylation of $\Delta^{4,12}$ and oxidation of C-11 *gem*-methyl into $CH_2OH$. HMBC spectra of compound **3** showed correlations between $H_2$-15 ($\delta_H$ 3.34)/C-11 ($\delta_C$ 38.0); C-15 ($\delta_C$ 71.7)/$H_2$-10 ($\delta_H$ 1.71, 1.42), and H-1 ($\delta_H$ 2.01). However, C-4 and C-5 showed similar heteronuclear interactions as in compound **2**. NOESY interactions of compound **3** supported the β orientations of both OMe-4 and $CH_2OH$-11 (Fig. 1). Moreover, NOED experiment showed 12.8% and 2.26% enhancement of β H-1 signal by the irradiations of β H-5 and $H_2$-15 signals, respectively. Similarly, irradiation of OMe signal enhanced H-5 signal (45%). Single-crystal X-ray diffraction technique was finally used to unambiguously deduce the structure as 4β-methoxycaryophyllene-5α, (11*R*)-15-diol (**3**) (Fig. 2).

Compound **4** was obtained as a white crystalline solid. HREI-MS showed the $M^+$ at *m/z* 254.1857 ($C_{15}H_{26}O_3$, calc. 254.1882), with the addition of two oxygen functions in the molecule, as compared to **1**. Presence of hydroxyl group was inferred by IR absorption at 3379 $cm^{-1}$. The $^1H$ NMR spectrum showed the disappearance of C-12 olefinic signals and the appearance of an additional $CH_2OH$ signal at $\delta$ 3.34 (*s*). The $^{13}C$ NMR spectra exhibited 15 carbon signals with two $-CH_3$, seven $-CH_2$, three –CH and three –C atoms. A new $CH_2$ signal at $\delta_C$ 71.2, along with the γ-up-field shifts of C-1 ($\delta_C$ 49.4) and C-10 ($\delta_C$ 23.5) signals, indicated the hydroxylation of C-11 *gem*-methyl group. Moreover, two additional signals appeared at $\delta_C$ 75.6 (C-4) and 21.9 (C-12), indicating the Markovnikov

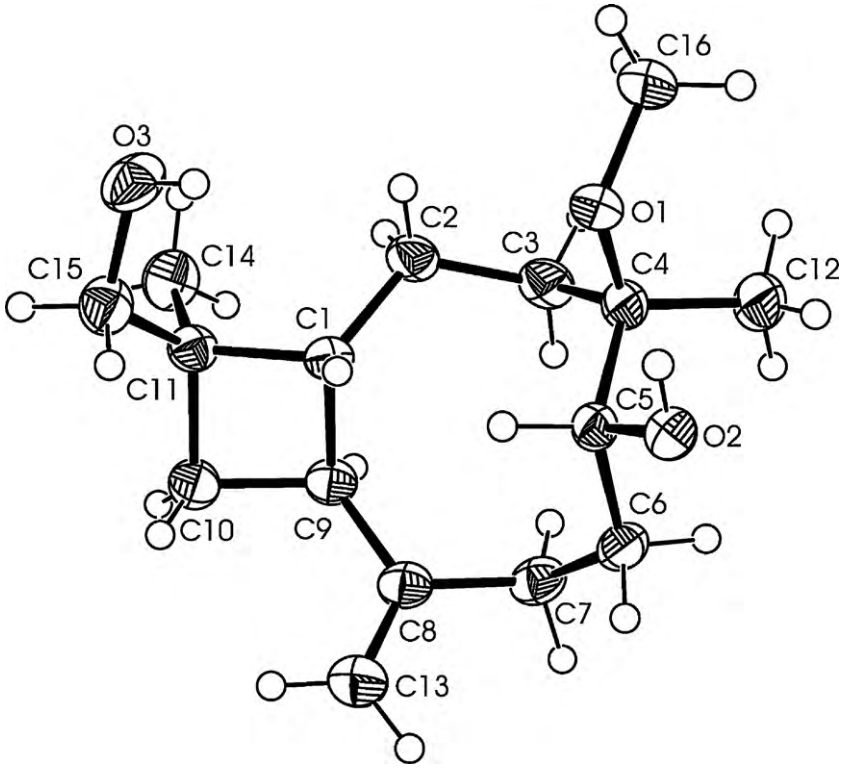



**Fig. 2.** ORTEP diagram of singe-crystal X-ray model of compound **3**.

hydration of C-4/C-12 double bound. Hydroxyl groups at C-4 and C-15 were further placed on the basis of HMBC interactions between H-5 ($\delta_H$ 3.65)/C-12 ($\delta_C$ 21.9), C-4 ($\delta_C$ 75.6), and C-7 ($\delta_C$ 30.2); $H_2$-15 ($\delta_H$ 3.34)/C-1 ($\delta_C$ 49.4) and C-11 ($\delta_C$ 38.5). The configuration of newly generated chiral centers, C-4 and C-11, were tentatively assigned as *R* on the basis of NOE experiments. NOESY spectra showed interactions between H-1β/H-5 and $H_2$-15, H-9α/$H_3$-14 and $H_3$-12. Furthermore, enhancement of H-1 signal (1.98%) by the irradiation of $H_2$-15 signal indicated the β-orientation of the $CH_2OH$ group at C-11. Similarly enhancement of α-oriented H-9 (2.08 and 4.88%) by the irradiation of $H_3$-12 and $H_3$-14α indicated the α-disposition of the $H_3$-12 group at C-4. Thus the structure of compound **4** was deduced as caryophyllene-5α, (11*R*)-15-diol.

Compounds **1**–**4** were also evaluated for *in vitro* antimalarial activity (*P. falciparum*, 3D7 strain). All transformed products, except **1**, were found to be active with $IC_{50}$ values of $3.09 \pm 2.61$ μg/ml, $0.72 \pm 0.17$ μg/ml, and $1.35 \pm 0.43$ μg/ml for compounds **2**, **3**, and **4**, respectively. The $IC_{50}$ value for positive control (chloroquine diphosphate) was $0.025 \pm 0.01$ μg/ml.

In conclusion, the biotransformation of 5α-hydroxycaryophylla-4(12), 8(13)-diene (**1**) with *M. phaseolina* yielded three polar oxidative metabolites. The biotransformation reaction involved hydroxylation at C-11 geminal methyls and regioselective Markovnikov addition on C-4/12 exocyclic double bond, while C-8/13 exocyclic double bond was found to be intact.

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