Microbial Conversion of Partheniol by *Calonectria decora*

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The biotransformation of partheniol by the fungus *Calonectria decora* ATCC 14767, produced six metabolites. These metabolites are; aromadendr-1(10)-en-8α -ol; 5(9),6-tricyclohumul-1(10)-en-8α-ol; 4,15-didehydro-6-bicyclohumulan-8α,10α-diol; 5(9),6-tricyclohumulan-4α,8α-diol; maalian-1α,8α-diol and 14-epi-1(4)-epoxymaalian-8α-ol. The identities of these metabolites were established by different spectroscopic measurements.

Introduction

Partheniol occurs in *Parthenium argentatum* × P. *tomentosa* Gray (guayule), Asteraceae, in a free form (Maatooq and Hoffmann, 1996) and as cinnamoyl ester (guayulin A) and *p*-methoxybenzoyl esters (guayulin B). Guayule is a natural rubber plant containing more than 10% resin with high abundance of these guayulins (Romo *et al.,* 1970; Proksch *et al.,* 1981; Schloman *et al.,* 1983; Banigan *et al.,* 1983). This made it possible to get gram quantities of these esters during our search for antifungal agents in the resin (Maatooq *et al.,* 1996). Partheniol was obtained by alkaline hydrolysis of guayulin B and was used as a substrate for our studies here. Partheniol possesses a cyclopropyl ring system and two endocyclic double bonds. Transannular cyclization or the Cope rearrangement (March, 1985; Brown and Foote, 1998; Vollhardt and Schore, 1999; Fox and Whitesell, 1997) can lead to different conformational derivatives as those observed with cyclodeca-1,5-dienes (Sorm, 1971; Takeda, 1974). The biotransformation can provide a good tool for getting such derivatives which could be more useful.

This paper describes the bioconversion of partheniol with *Calonectria decora* ATCC 14767, into six different metabolites. The fermentation, isolation and structural identification of these metabolites are discussed.

Results and Discussion

For the biotransformation of partheniol (**1**), several bacteria and fungi were subjected to screening experiments. It was found that *Calonectria decora* ATCC 14767 was able to convert partheniol into several metabolites. Scale up of this reaction afforded the isolation of these metabolites (**2–7**).

The GC-EI-MS of metabolite **2** gave *m/z* of 202 analyzed for $[M-H_2O]^+$, which indicated a molecular formula of $C_{15}H_{24}O$. The two olefinic carbon signals at δ 123.1 and 139.6 proved that only one double bond at 1(10)-position is still intact. However, these two olefinic carbon signals are quater-

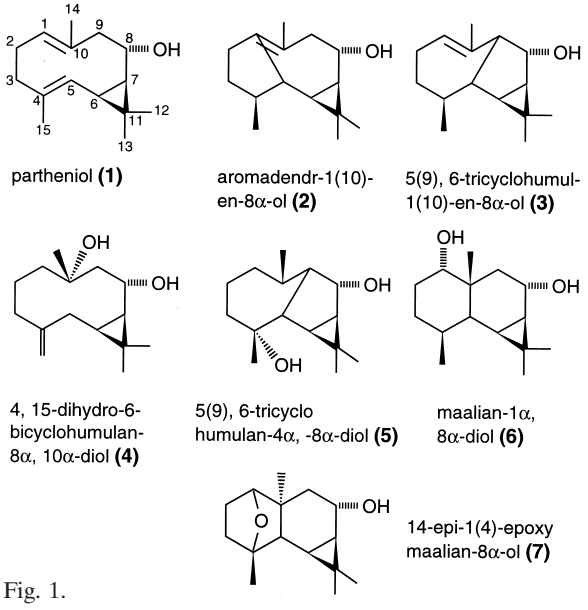

Fig. 1.

naries, which indicated the likely involvement of 1-position in a new ring formation. The methyl group proton doublet at δ 0.94 (J = 8 Hz) which was assigned to position-15 excluded the involvement of C-4 in the new ring formation. The appearance of H-6 as a triplet at δ 0.70, indicated the presence of a methine group proton at C-5, which supported the involvement of C-5 in the new ring formation with C-1. The downfield methyl proton singlet at δ 1.64, which is correlated to the carbon signal at δ 15.3 was assigned to C-14. The relative stereochemistry of 15-methyl group was assigned to be β-oriented based on its ^{13}C-NMR chemical shift value (δ 21.9) which is consistent with its aromadendrane analogs (Atta-Ur-Rahman and Ahmad, 1992; Canigueral *et al*.,1994). These evidences proved that the structure of metabolite **2** is aromadendr-1(10)-en-8α-ol.

Metabolite **3** possesses a spectroscopic data close to those of metabolite **2** with few differences. The H-8 proton signal appeared as a double doublet (δ 3.99, J = 10, 6 Hz), pointing to the presence of a methine group proton at C-9, which indicated its likely involved in a new bridge formation. The 15-methyl group protons signal remains as a doublet and H-6 is a triplet indicating that position-5 is the other side of the bridge. The DEPT spectrum showed a quaternary carbon signal at δ 141.1 and an olefinic methine carbon signal at δ 114.4 (correlated to a broad proton triplet at δ 5.31) were assigned to the double bond at 1(10)-position. These findings proved that the structure of metabolite **3** is 5(9),6-tricyclo-humul-1(10)-en-8α-ol.

Table I. ^{13}C-NMR data of partheniol and its metabolites (**2–7**)*.

C#	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	126.9	123.1	114.4	39.1	36.3	74.6	83.2
2	25.3	29.7	28.8	32.1	26.4	29.3	38.2
3	46.3	26.2	40.1	47.6	41.1	56.0	53.3
4	129.2	37.3	43.4	148.8	73.4	41.2	86.2
5	125.3	41.1	50.5	42.3	48.6	57.2	60.2
6	36.0	32.3	30.3	34.1	32.0	35.3	32.3
7	28.8	28.3	21.1	32.3	28.8	29.1	26.2
8	72.6	67.7	64.4	69.9	65.8	67.5	66.0
9	40.5	32.4	52.1	42.2	57.5	38.1	49.4
10	136.9	139.6	141.1	81.8	39.8	35.7	36.7
11	20.5	18.4	20.2	19.8	19.4	20.8	19.8
12	29.2	28.1	29.2	28.5	29.9	29.2	29.1
13	16.5	15.5	16.1	17.4	16.0	16.0	16.4
14	15.5	15.3	20.8	18.8	16.7	16.5	24.3
15	20.8	21.9	23.8	109.2	21.4	23.5	25.8

* At 62.5 MHz, using $CDCl_3$ as a solvent (except 4, **5** in 1:1, $CDCl_3$–CD_3OD), TMS is the internal standard and the chemical shifts (δ) are expressed in ppm.

Table II. ^{1}H-NMR data of partheniol and its metabolites (**2–7**)*.

H#	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	4.91 *dd*, 6, 9	–	5.31 br t, 4	–	–	3.35 *dd*, 6, 7	3.24 *dd*, 6, 8
5	4.36 *d*, 11	–	–	–	–	–	–
6	1.42 *dd*, 11, 9	0.70 *t*, 10, 9	0.78 *t*, 10, 9	0.74 *m*	0.19 *t*, 10, 9	0.61 *t*, 10, 9	0.12 *t*, 10, 9
7	0.73 *dd*, 11, 9	0.65 *t*, 10, 9	0.68 *t*, 10, 9	0.62 *t*, 10, 9	0.71 *t*, 10, 9	0.68 *t*, 10, 9	0.62 *t*, 10, 9
8	3.60 *ddd*, 10, 10, 5	4.01 *m*	3.99 *dd*, 10, 6	*m*	3.75 *t, 9, 5*	3.73 *m*	3.68 *m*
12	1.12 *s*	1.09 *s*	1.12 *s*	1.11 *s*	1.08 *s*	1.11 *s*	1.02 *s*
13	1.19 *s*	1.16 *s*	1.18 *s*	1.14 *s*	1.09 *s*	1.11 *s*	1.03 *s*
14	1.44 *s*	1.64 *s*	0.93 *s*	1.15 *s*	0.95 *d,7*	1.18 *s*	1.08 *s*
15	1.63 *s*	0.94 *d*, 8	1.31 *d*, 8	4.82 *dd*, 1, 1	1.15 *s*	0.91 *d*, 7	1.21 *s*

* At 250 MHz, using $CDCl_3$ as a solvent (except **4, 5** in 1:1, $CDCl_3$–CD_3OD), TMS is the internal standard, the chemical shifts (δ) are expressed in ppm and the coupling constant J in Hz. d = doublet, dd = double doublets, m = multiplet, t = triplet and s = singlet, br = broad.

Metabolite **4** gave *m/z* 238 analyzed for $C_{15}H_{26}O_2$. The two carbon signals at δ 119.2 and 148.8 together with the pair of proton doublets at δ 4.82 (2H, J = 1 Hz each), indicated the presence of an exomethylene group. Position 4(15) is the only possible place for that exomethylene. DEPT spectrum concluded a quaternary carbon signal at δ 81.8 which is good value for an α-hydroxylated C-10 in a humulane skeleton (Atta-Ur-Rahman and Ahmad, 1992). This indicated that metabolite **4** should be 4,15-didehydro-6-bicyclohumulan-8α,10α-diol (6-bicyclohumul-4(15)-8 α,10α -diol).

The GC-EI-MS of metabolite **5** gave *m/z* of 220 analyzed for $[M-H_2O]^+$, which concluded a molecular formula $C_{15}H_{26}O_2$. The ^{1}H-NMR spectrum demonstrated three characteristic triplets at δ 0.19, 0.71 (J = 10, 9 Hz each) and 3.75 (J = 9, 5 Hz), assigned to one proton each at 7, 6 and 8-positions, respectively. This referred to the likely presence of C-5 and C-9 as methine groups, which mean a possible bridge formation between C-5 and C-9 to give 5(9), 6-tricyclohumulane skeleton. The appearance of 14-methyl group as a doublet at δ 0.95, excluded the possible bridge formation at C-10. The relative stereochemistry of 15-methyl group was assigned to be β-oriented based on analogy with other metabolites obtained with a different bug (*Mucor circinelloides*) in our laboratory (Maatooq, 2002). The quaternary carbon signal at δ 73.4 (DEPT) was assigned to an α-hydroxylated C-4 (Atta-Ur-Rahman and Ahmad, 1992). These data concluded that the structure of metabolite **5** is 5(9),6-tricyclohumulan-4α,8α-diol.

Metabolite **6** EI-MS gave *m/z* of 238 corresponding to $C_{15}H_{26}O_2$. The ^{1}H-NMR showed two triplets at δ 0.61 and 0.68 assigned to H-6 and H-7, respectively, which indicated the possible presence of a methine group at C-5. The multiplet at δ 3.73 assigned to H-8, indicated that a methylene group is present at C-9. The proton singlets at δ 1.11, 1.11 and 1.18 were assigned to 12, 13 and 14-methyl groups, respectively. The doublet at δ 0.91 (3H) was assigned to a 15-methyl group, which suggested a linkage between C-5 and C-10. The double doublet δ 3.35 was correlated to the carbon signal at δ 74.6 and was assigned to an α-hydroxylated position-1 in maaliane skeleton (Atta-Ur-Rahman and Ahmad, 1992). The relative stereochemistry of 14 and 15-methyl groups was assigned to be β-oriented based on comparison of their NMR data with a series of metabolites, having maaliane skeleton obtained with another bug (*Mucor circinelloides*) in our laboratory (Maatooq, 2002). Therefore, the relative structure of metabolite **6** has to be maalian-1α,8α-diol.

Metabolite **7** EI-MS gave *m/z* of 236 calculated for $C_{15}H_{24}O_2$. The ^{13}C-NMR spectrum showed no olefinic signals. The ^{1}H-NMR spectrum showed two upfield triplets at δ 0.12 and 0.63 assigned to H-6 and H-7, respectively, and indicated the possible presence of a methine group at C-5. This indicated the likely involvement of C-5 in a new linkage formation. The four, 3H, singlets at δ 1.02, 1.03, 1.08 and 1.21 were assigned to 12, 13, 14 and 15-methyl groups, respectively. The shielding of the methyl groups confirmed the loss of the two double bonds at C-1(10) and C-4. The appearance of 14-methyl proton signal as a singlet made it possible to predict that C-10 has to be involved in the new linkage with C-5 in a transannular cyclization. The carbon signal at δ 86.2 was assigned to a quaternary carbon either at 4 or 10-position. The relative deshielding effect on 15-methyl group linked this δ 86.2 value to C-4. The proton signal at δ 3.24 (*dd*, J = 6, 8 Hz) was assigned to H-1 based on its correlation to the carbon signal at δ 83.2. Since the molecular weight of metabolite **7** is 236, which pointed to two mass units loss, this could be corrected by a linkage formation between C-1 and C-4 to form a tetrahydrofuran ring system. Moreover, the chemical shift values of δ 86.2 and 83.2 are in agreement with 2, 5-disubstituted tetrahydrofuran derivatives (Rodriguez-Hahn *et al.,* 1970; Komoroski *et al.,* 1986). The stereochemistry of C-14 and C-15 were assigned to be α and β, respectively, based on direct comparison with **6** and other metabolites obtained with another bug (*Mucor circinelloides*) in our laboratory (Maatooq, 2002). For these reasons, the relative structure of **7** has to be 14-epi-1(4)epoxymaalian-8α-ol.

In conclusion, six partheniol metabolites (**2–7**) were isolated from the biotransformation reactions with *Calonectria decora* ATCC 14767. Several enzymatic systems seem to be involved in these reactions, where hydroxylation, epoxidation and transannular cyclization were evidenced. Dioxygenation is not detected while monooxygenation was observed (**4–7**) and a new double bond formation was evidenced (**4**). The microorganism demonstrated its ability to induce transannular

cyclization at positions1–5 (aromadendrane skeleton, **2**), 5–9 [5(9),6-tricyclohumulane skeleton, **3** and **5**] and 5–10 (maaliane skeleton, **6** and **7**) positions. The 5–9 cyclization looks unusual and unexpected, however, 9-position was proved to be hydroxylated by *Mucor circinelloides* (Maatooq, 2002). This indicated that 9-position is enzymatically accessible and this hydroxylation might be the first step for the subsequent cyclization.

Experimental

General instrumentation

Melting points are uncorrected. ^{1}H-NMR and ^{13}C-NMR were measured on a Bruker WM 250 NMR spectrometer, at 250 MHz and 62.5 MHz, respectively, with $CDCl_3$ or 1:1 $CDCl_3$–CD_3OD as the solvent and TMS as the internal standard. The chemical shifts are expressed in δ (ppm). DEPT (discriminate the carbon signals into CH_3, CH_2 and CHs, while the quaternaries are obscured) and HETCOR (direct C–H correlation) were measured on a Bruker WM 300 NMR spectrometer. EI-MS and GC-EI-MS were conducted at 70 eV using a 25 meter HP-5 capillary column, 0.2 mm ID, film thickness 0.33 μm, cross-linked 5% phenyl-methyl silicone, helium with head pressure of 18 psi, 1 μl injection, split ratio 1:50; injector 200°, detector 300°, temperature program was 70°, hold for 1 min, 20°/min. IR was conducted on Beckman Acculab I IR spectrometer. Optical rotations were measured on Autopole III Automatic polarimeter (Rudolph Scientific, Fairfield, New Jersey).

Substrate material

Partheniol was obtained by alkaline hydrolysis of guaylin-B obtained from the resin of *Parthenium argentatum* × *P. tomentosa* Six grams of guayulin-B were dissolved in 600 ml 10% NaOH/MeOH, and the mixture was refluxed for two hours. The reaction was terminated by reducing the volume to 100 ml under vacuum then 500 ml of water was added and extracted with EtOAc 3 × 600 ml. After drying over anhydrous Na_2SO_4 followed by solvent evaporation under vacuum, 3.1 g were recovered. Final purification was conducted by flash column chromatography, 200 g Si gel, 63–200 μ, 3.5 × 45 cm. Isocratic elution with 10% EtOAc/hexane, was adopted to give 2.8 g needles of partheniol. The identity of partheniol was confirmed cochromatographically (R_f = 0.23 in 10% EtOAc/hexane, Si gel GF_{254} TLC plates) and by ^{1}H and ^{13}C-NMR and mass spectrometry (Parodi *et al.,* 1987; Maatooq and Hoffmann, 1996).

Fermentation methods

Several cultures were grown according to the standard two-stages fermentation protocol (Betts *et al.,* 1974). Screening experiments were done in 125 ml DeLong culture flasks. The culture flasks held one fifth of their volume of the following medium; 2% glucose, 0.5% soybean meal, 0.5% yeast extract, 0.5% NaCl and 0.5% K_2HPO_4. The pH of the medium was adjusted at 7.0 using 6 N HCl before autoclaving for 20 m at 121° and 15 psi. After inoculation with the slants, stage I cultures (in which the microorganism was transferred from the slants to the sterile medium) were incubated at 27° and 250 rpm for 72 h before being used to inoculate stage II culture flasks (in which, 10% inoculum volumes of stage I culture was used to inoculate another sterile medium and leave for 24 h to give this stage). For screening scale experiments 10 mg of partheniol in 0.1 ml of 1:1 dimethylformamide (DMF)- dichloromethane (DCM) mixture was added to 24-h old stage II cultures, which were incubated again and sampled periodically for analysis.

Sampling

Samples of 1 ml each were taken after 12, 24, 36 and 48 h and every other day for 2 weeks following substrate addition. Each sample was extracted by shaking with 0.5 ml EtOAc and spun at 3000 × *g* for 1 m in a desk-top centrifuge. All the samples EtOAc extract were spotted on Si gel GF_{254} TLC plates, and developed with a suitable percentage of EtOAc/hexane or acetone/DCM, and visualized after spraying with 0.001% vanillin/H_2SO_4 followed by heating for 5–10 s with a heat gun. It was found that *Calonectria decora* ATCC 14767, was able to convert partheniol into several metabolites.

Preparative scale conversion of partheniol

Thirty two 125 ml stage II culture flasks of *Calonectria decora* ATCC 14767, received 800 mg of

partheniol in 3.2 ml of 1:1 DMF-DCM mixture (1 mg substrate per ml of culture medium). After incubation for 18 days under the usual condition, the cultures were combined and exhaustively extracted with 3 × 1.5 liter of 10% MeOH/EtOAc. The extract was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield a crude dark oily residue of 1.15 g.

Isolation and purification

The reaction extract (1.15 g) was column chromatographed by flash method, 200 g Si gel 63–200 μ, 2.2 × 45 cm the elution was achieved by EtOAc/hexane using 1-liter volume each of 10%, 15%, 20%, 25%, 35%, 50% and 100%. Twenty two fractions (250 ml each) were collected. After TLC in a suitable solvent system, similar fractions were pooled. This afforded four groups of fractions.

Frs. 3–4 (110 mg) contain the recovered unreacted substrate.

Frs. 5–6 (78 mg) were purified by prep. TLC using 25% EtOAc/hexane as a solvent system (R_f = 0.53). This gave 14 mg of **2** as a solid gum. Frs. 11–18 (230 mg) were resolved into two compounds by prep. TLC using 50% EtOAc/hexane as a solvent system (R_f = 0.48 and 0.46). This gave 22 mg of **3** as needles and 44 mg of **4** as a solid gum.

Frs. 19–21 (506 mg) were fractionated by MPLC, 140 g Si gel, 15–25 μ, 26 × 460 mm. The elution solvent was EtOAc/hexane using 500 ml portions of 50%, 60%, 70%, 80%, 90% and 100%. Twenty fractions, 150 ml each, were collected. Sub-fraction 5 (105 mg) was purified on prep. TLC, using 50% acetone/hexane as a solvent (R_f = 0.68). This gave 70 mg of **5** as needles. Sub-fraction 6 (48 mg), after prep. TLC using the 50% acetone/hexane solvent system (R_f = 0.53) gave 29 mg of **6** as a solid gum. The sub-fractions 15–17 (62 mg) were treated same way as sub-fraction 6 to give 22 mg of **7** as a solid gum (R_f = 0.21).

Metabolite **2**, *(+)aromadendr-1(10)-en-8α-ol*

Solid gum, $\alpha[D]^{25}$, +14.4 ($CHCl_3$; c. 0.75), IR $\upsilon_{max}^{cm^{-1}}$; 3390, 2950, 2900, 2840, 1640, 1440, 1370, 1260, 1040, 1000 and 730. GC-EI-MS, 70 eV, *m/z* (relative intensity); 202 $[M-H_2O]^+$ (32), 187 $[M-H_2O-CH_3]^+$ (20), 159 (21), 136 (34), 123 (22), 121 (72), 119 (32), 107 (4), 85 (62), 55 (40), 41 (100) and 28 (90). The ^{13}C and ^1H-NMR data of metabolites **2–7** are presented in Tables I and II, respectively.

Metabolite **3**, *(+)5(9),6-tricyclohumul-1(10)-en-8α-ol*

Solid gum, $\alpha[D]^{25}$, +11.4 ($CHCl_3$; c. 0.75), IR $\upsilon_{max}^{cm^{-1}}$; 3395, 2920, 2870, 1630, 1450, 1370, 1260, 1170, 1100, 1040, 990, 920 and 870. GC-EI-MS, 70 eV, *m/z* (relative intensity); 220 $[M]^+$ (8), 205 $[M-CH_3]^+$ (12), 187 $[M-H_2O-CH_3]^+$ (8), 177 (21), 147 (22), 133 (12), 121 (18), 109 (28), 107 (30), 93 (24), 91 (30), 59 (30) and 43 (100).

Metabolite **4**, *(−)4,15-didehydro-6-bicyclohumulan-1(10)-en-8α,10α-diol*

Solid gum, $\alpha[D]^{25}$, −4.8 ($CHCl_3$; c. 0.10), IR $\upsilon_{max}^{cm^{-1}}$; 3400, 2960, 2920, 2840, 1620, 1450, 1370, 1230, 1120 and 1020. GC-EI-MS, 70 eV, *m/z* (relative intensity); 238 $[M]^+$ (4), 220 $[M-H_2O]^+$ (10), 205 $[M-H_2O-CH_3]^+$ (10), 111 (34),107 (45), 55 (60), 43 (96) and 41(100).

Metabolite **5**, *(+)5(9),6-tricyclohumulan-4α,8α-diol*

Needles, m.p. 172° , $\alpha[D]^{25}$, +1.2 ($CHCl_3$; c. 2.00), IR $\upsilon_{max}^{cm^{-1}}$; 3360, 2940, 2860, 1450, 1370, 1160, 1120, 1100, 1040 and 1020. GC-EI-MS, 70 eV, *m/z* (relative intensity); 220 $[M-H_2O]^+$ (6), 205 $[M-H_2O-CH_3]^+$ (10), 187 $[M-2H_2O-CH_3]^+$ (6), 177 (12), 149 (13), 121 (18), 107 (24), 95 (20), 81 (22), 43 (100) and 41 (44).

Metabolite **6**, *(−)maalian-1α,8α-diol*

Solid gum, $\alpha[D]^{25}$, −7.6 ($CHCl_3$; c. 1.25), IR $\upsilon_{max}^{cm^{-1}}$; 3380, 2950, 2860, 1445, 1370, 1260, 1115, 985, 1020, 970, 930 and 730. GC-EI-MS, 70 eV, *m/z* (relative intensity); 238 $[M]^+$ (2), 220 $[M-H_2O]^+$ (6), 205 $[M-H_2O-CH_3]^+$ (5), 202 $[M-2H_2O]^+$ (10), 177 (12), 121 (15), 109 (18), 107 (19), 81 (20), 55 (26), 43 (100) and 41 (42).

Metabolite **7**, *(+)14-epi-1(4)-epoxymaalian-8α-ol*

Solid gum, $\alpha[D]^{25}$, +11.8 ($CHCl_3$; c. 0.75). IR $\upsilon_{max}^{cm^{-1}}$; 3350, 3020, 2950, 2850, 1450, 1320, 1265, 1160, 1110, 1040, 1020, 970, 920, 820 and 730. GC-EI-MS, 70 eV, *m/z* (relative intensity); 236 $[M-H_2O]^+$ (2), 221 $[M-H_2O-CH_3]^+$ (6), 218 $[M-2H_2O]^+$ (8), 203 $[M-2H_2O-CH_3]^+$ (6), 175 (8), 160 (10), 145 (8), 125 (6), 121 (6), 107 (12), 93 (11), 69 (7), 55 (13), 43 (100) and 41 (30).

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