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ORIGINAL ARTICLE

Hydroxylation of (+)-menthol by *Macrophomina phaseolina*

SYED GHULAM MUSHARRAF, MUHAMMAD ARIF AHMED, RAHAT AZHER ALI & MUHAMMAD IQBAL CHOUDHARY

H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

Abstract

Biotransformation of (+)-menthol with *Macrophomina phaseolina* led to hydroxylations at C-1, C-2, C-6, C-7, C-8 and C-9, with the C-8 position being preferentially oxidized. The resulting metabolites were identified as 8-hydroxymenthol (**2**), 6*R*-hydroxymenthol (**3**), 1*R*-hydroxymenthol (**4**), 9-hydroxymenthol (**5**), 2*R*,8-dihydroxymenthol (**6**), 8*S*,9-dihydroxymenthol (**7**), 6*R*,8-dihydroxymenthol (**8**), 1*R*,8-dihydroxymenthol (**9**) and 7,8-dihydroxymenthol (**10**). Metabolites **6**–**10** are described here for the first time. Their structures were characterized by spectroscopic analysis.

Keywords: *Macrophomina phaseolina*, biotransformation, (+)-menthol, hydroxylation

Introduction

Microbial transformation of terpenes is of increasing interest and new metabolites with unique structural features and enhanced biological activity have been produced (Aranda et al. 1991; Maurs et al. 1999; Abraham et al. 2000). The potential for insertion of a hydroxyl group in a single step with high regio- and stereoselectivity at unactivated carbons is a major advantage of microbial transformation (Holland 1982) and represents an inexpensive route to asymmetric building blocks that can be used as hemisynthesis intermediates, chiral auxiliaries and chiral synthons for asymmetric synthesis (Azerad 2000; de Carvalho & da Fonseca 2006).

Menthol is an abundantly available bioactive monoterpene from the natural chiral pool. Menthol and its derivatives have wide applications in synthetic organic chemistry, particularly in asymmetric synthesis (Oertling et al. 2007), because of their non-toxic, environmental friendly and crystalline nature. They are also available in bulk quantities in both enantiomeric (±) forms at low cost. Biotransformation of (−)-menthol has already been studied (Shukla et al. 1987; Tsutomu et al. 1989; Yoshinori et al. 1991; Figueiredo et al. 1996; Atta-ur-Rahman et al. 1998; Mitsuo et al. 1999, 2003a,b; Mohammadreza et al. 2005), but there are few reports of biotransformation of (+)-menthol (**1**) (Mitsuo et al. 1999; Yoshinori et al. 1991; Yutaka et al. 1991).

In continuation of our work on biotransformation of bioactive compounds (Choudhary et al. 2006a,b,c), we describe here the biotransformation of (+)-menthol (**1**) by *Macrophomina phaseolina*, which had previously been screened with various terpenoidal compounds and showed interesting structural modification of the parent compounds (Choudhary et al. 2006a; Musharraf et al. 2010). Some potential *p*-menthane-diol products, such compounds **2** and **5**, are relevant industrial products with applications as flavors and fragrances (Kenmochi et al. 1999).

Methods

General experimental method

(+)-Menthol (**1**) was purchased from Sigma Aldrich (USA). TLC used plates pre-coated with silica gel

Correspondence: S. G. Musharraf, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi – 75270, Pakistan. Tel: +92-321-4824924. Fax: +92 321-4819018/9. E-mail: musharraf1977@yahoo.com

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(PF_{254}, 20 × 20 cm, 0.25 mm; Merck) and column chromatography used silica gel (70–230 mesh; Merck, USA). Melting points were determined using a Buchi-535 melting point apparatus (Swizerland); optical rotations in MeOH solutions used a Jasco DIP-360 digital polarimeter (Tokyo, Japan); IR spectra in cm^{-1} in $CHCl_3$ solutions used an FTIR-8900 spectrophotometer (Shimadzu, Japan). ^1H and ^{13}C NMR spectra in $CDCl_3$ solutions used a Bruker Avance 400 NMR spectrometer (Swizerland) at 400 and 100 MHz; ^2D experiments with $CDCl_3$ solutions used the same instrument. Chemical shifts (δ) are given in ppm relative to $SiMe_4$ as an internal standard; coupling constants $\mathcal{J}$ are in Hz. EI-MS and HRFAB-MS used a Jeol JMS-600H mass spectrometer (Japan) in *m/z* (rel. %). Compounds were detected by TLC with the help of vanillin spray reagent.

Organisms and culture media

Stock cultures of *M. phaseolina* (KUCC 730) were maintained at 4°C on agar plates (Sabouraud dextrose agar). The medium for *M. phaseolina* was prepared by mixing the following ingredients in 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) and NaCl (15.0 g).

Fermentation of (+)-menthol (1) with M. phaseolina

The fermentation medium was distributed into 30 flasks of 250 mL capacity (100 mL in each) and autoclaved at 121°C for 20 min. The substrate (600 mg) was dissolved in acetone (15 mL) and the resulting solution evenly distributed among the 30 flasks containing 24-h-old stage II cultures of *M. phaseolina* (20 mm culture/flask) and fermentation continued for 12 days on a shaker (200 rpm) at 29 ° C. During the fermentation period, aliquots were taken out from the culture daily and analyzed by TLC in order to determine the degree of transformation of the substrate. In all experiments, one control flask without fungus (for checking 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 L) and the filtrate was extracted with CH_2Cl_2 (3 × 1.5 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.

After filtration, extraction and evaporation, a brown gum (1.32 g) was obtained which after repeated column chromatography with a gradient mixture of petroleum ether–EtOAc yielded **1** (21.3 mg; with petroleum ether–EtOAc 9:1), **2** (44.6 mg; with petroleum ether–EtOAc 8.6:1.4), **3** (15.1 mg; with petroleum ether–EtOAc 8.4:1.6), **4** (33.5 mg; with petroleum ether–EtOAc 8.1:1.9), **5** (13.2 mg; with petroleum ether–EtOAc 8:2), **6** and **7** (15.3 mg; with petroleum ether–EtOAc 7.6:2.4), **8** (8.1 mg; with petroleum ether–AcOEt 7.1:2.9), **9** (6.8 mg; with petroleum ether–EtOAc 7:3) and **10** (9.4 mg; with petroleum ether–EtOAc 6.7:3.3). An impure fraction containing compounds **6** and **7** was purified by TLC on pre-coated plates using petroleum ether (EtOAc) (7.9:2.1) as mobile phase where compounds **6** (6.4 mg) and **7** (5.2 mg) were obtained.

Compound **6** was obtained as a colorless viscous liquid. $[\alpha]_D^{25}$: +31.4° (*c* = 0.11, $CHCl_3$). IR ($CHCl_3$), ν_{max} (cm^{-1}): 3381, 1472, 1388. For ^1H and ^{13}C NMR spectroscopic data, see Table I. EI-MS, *m/z* (rel. %): 173 ($[M - Me]^+$) (4); 170 ($[M - H_2O]^+$) (2); 155 ($[M - Me - H_2O]^+$) (9); 137 (15); 112 (100); 97 (53). HRFAB-MS (+ve), *m/z*: 189.1422. Calcd for $C_{10}H_{21}O_3$: 189.1491.

Compound **7** was obtained as a colorless viscous liquid. $[\alpha]_D^{25}$: +50.1° (*c* = 0.21, $CHCl_3$). IR ($CHCl_3$), ν_{max} (cm^{-1}): 3412, 1462, 1032. For ^1H and ^{13}C NMR spectroscopic data, see Table I. EI-MS, *m/z* (rel. %): 157 ($[M - CH_2OH]^+$) (24); 139 (38); 109 (11); 95 (44); 81 (100); 55 (70). HRFAB-MS (+ve), *m/z*: 189.1479. Calcd for $C_{10}H_{21}O_3$: 189.1491.

Compound **8** was obtained as a white crystalline solid, mp 91–92°C. $[\alpha]_D^{25}$: +27.4° (*c* = 0.51, $CHCl_3$). IR ($CHCl_3$), ν_{max} (cm^{-1}): 3441, 1464, 1382, 1022. For ^1H and ^{13}C NMR spectroscopic data, see Table I. EI-MS, *m/z* (rel. %): 173 ($[M - Me]^+$) (4); 175 ($[M - H_2O]^+$) (2); 155 ($[M - Me - H_2O]^+$) (9); 137 (15); 112 (100); 97 (53). HRFAB-MS (+ve), *m/z*: 189.1399. Calcd for $C_{10}H_{21}O_3$: 189.1491.

Compound **9** was obtained as a white crystalline solid, mp 85–86°C. $[\alpha]_D^{25}$: +61.4° (*c* = 0.81, $CHCl_3$). IR ($CHCl_3$), ν_{max} (cm^{-1}): 3318, 1469, 1374. For ^1H and ^{13}C NMR spectroscopic data, see Table I. EI-MS, *m/z* (rel. %): 173 ($[M - Me]^+$) (5); 170 ($[M - H_2O]^+$) (3); 165 ($[M - Me - H_2O]^+$) (13); 137 (16); 112 (31); 94 (91); 87 (59). HRFAB-MS (+ve), *m/z*: 189.1411. Calcd for $C_{10}H_{21}O_3$: 189.1491.

Compound **10** was obtained as a colorless viscous liquid. $[\alpha]_D^{25}$: +83.7° (*c* = 0.75, $CHCl_3$). IR ($CHCl_3$), ν_{max} (cm^{-1}): 3374. For ^1H and ^{13}C NMR spectroscopic data, see Table I. EI-MS, *m/z* (rel. %): 173 ($[M - Me]^+$) (3); 155 (15); 94 (87); 79 (100); 59 (32). HRFAB-MS (+ve), *m/z*: 189.1438. Calcd for $C_{10}H_{21}O_3$: 189.1491.

Table I. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of compounds **6** – **10** in $CDCl_3$.

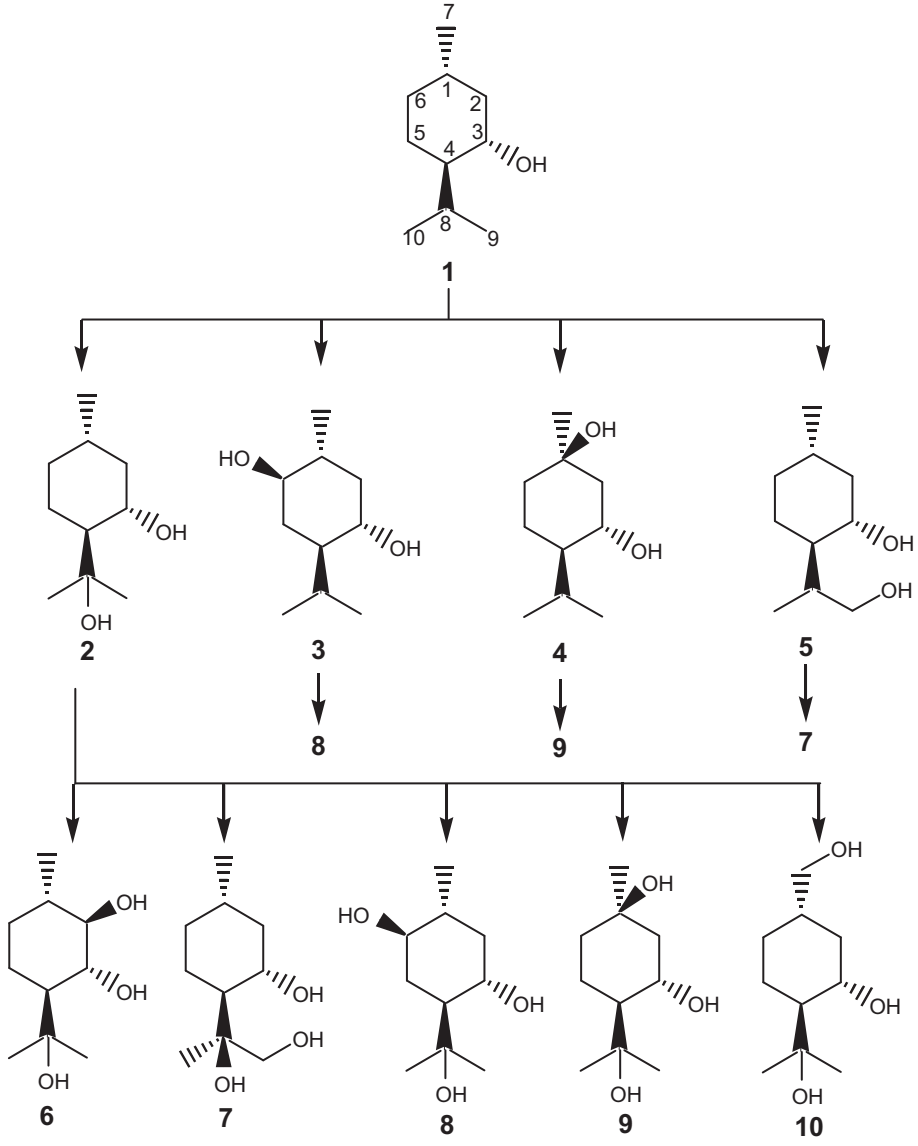
	6		**7**		**8**		**9**		**10**	
Position	δ_H (ppm) ($\mathcal{J}$ in Hz)	δ_C (ppm)	δ_H (ppm) ($\mathcal{J}$ in Hz)	δ_C (ppm)	δ_H (ppm) ($\mathcal{J}$ in Hz)	δ_C (ppm)	δ_H (ppm) ($\mathcal{J}$ in Hz)	δ_C (ppm)	δ_H (ppm) ($\mathcal{J}$ in Hz)	δ_C (ppm)
1	1.51, m	37.2	1.28, m	32.6	1.48, m	39.1	–	71.5	1.51, m	40.4
2	3.04, t (9.6)	81.1	1.89, m	43.6	1.79, m	43.3	1.92, dd (2.8, 4.4)	48.6	2.02, m	39.8
			1.46, m		1.36, m		1.40, m		1.04, m	
3	3.47, t (9.7)	77.4	3.78, dt (4.2, 10.5)	73.2	3.70, dt (4.3, 10.5)	73.0	4.06, dt, (4.4, 10.3)	70.5	3.74, dt (4.3, 10.4)	73.7
4	1.41, m	51.3	1.42, m	53.5	1.42, m	52.7	1.38, m	54.2	1.41, m	54.6
5	1.65, m	26.4	1.74, m	27.3	1.81, m	36.8	1.51, m	23.6	1.82, m	27.4
	1.12, m		0.98, m		1.37, m		1.34, m		1.01, m	
6	1.69, m	32.2	1.64, m	35.5	3.10, dt (4.3, 10.4)	76.3	1.61, m	39.1	1.78, m	30.1
	0.99, m		1.50, m				1.38, m		0.94, m	
7	1.02, d (6.4)	18.0	0.91, d (6.51)	22.4	0.91, d (6.4)	18.6	1.25, s	24.3	3.47, 2H, br. d (6.2)	67.9
8	–	74.4	–	76.9	–	75.1	–	75.6	–	75.5
9	1.20, s	30.0	3.73, d (11.2)	68.7	1.17, s	29.5	1.24, s	31.2	1.21, s	29.6
			3.42, d (11.2)							
10	1.22, s	23.8	1.20, s	23.3	1.20, s	24.2	1.23, s	29.5	1.21, s	24.1

Results and discussion

Incubation of compound **1** ($C_{10}H_{20}O$) with *M. phaseolina* for 12 days resulted in the production of two sets of metabolites (Scheme 1): monohydroxylated derivatives **2**–**5** and dihydroxylated derivatives **6**–**10**. Compounds **2**–**5** are all known compounds, reported as biotransformed products of compound **1** by *Aspergillus niger* (Yoshinori et al. 1991), while all dihydroxylated derivatives **6**–**10** were found to be new compounds. Structures of metabolites were elucidated through comparative spectroscopic studies with substrate **1** and comparison with reported data (Yoshinori et al. 1991); however, for compound **5** the stereochemistry at C-8 remains to be clarified.

The EI-MS of compound **6** showed the $[M - Me]^+$ and $[M - H_2O]^+$ ions at m/z = 173 and 170, respectively. The HRFAB-MS (+ve) exhibited the $[M + H]^+$ ion at m/z = 189.1422 corresponding to the formula $C_{10}H_{21}O_3$, consistent with a dihydroxy derivative of **1**. The ^{1}H NMR spectrum of **6** ($CDCl_3$, 400 MHz) exhibited a new downfield methine signal at δ = 3.04 ppm (t, $\mathcal{J}_{2ax,1ax/3ax}$ = 9.6 Hz), while H-3 also appeared as a clear triplet at δ = 3.47 ppm ($\mathcal{J}_{3ax,2ax/4ax}$ = 9.7 Hz). This suggested the presence of an additional OH group at the C-2 position. In addition to this, C-8 geminal secondary methyls appeared as singlets, instead of doublets, at δ = 1.20 and 1.22 ppm. This indicated hydroxylation at a C-8 tertiary carbon. The ^{13}C NMR spectrum of **6** showed two new signals at δ = 81.1 and 74.4 ppm, corresponding to C-2 and C-8, respectively. The COSY 45° spectrum had interactions of H-1 (δ = 1.51 ppm) and H-3 (δ = 3.47 ppm) with the newly oxygenated methine H-2 (δ = 3.04 ppm), while the HMBC spectrum showed interaction of H_3-7 (δ = 1.02 ppm) and H-3 (δ = 3.47 ppm) with C-2 (δ = 81.1 ppm). The H_3-9 (δ = 1.20 ppm) and H_3-10 (δ = 1.22 ppm) showed HMBC with C-8 (δ = 74.4 ppm), which further suggested the presence of a hydroxyl group at C-8. The multiplicity of the H-2 signal at δ = 3.04 ppm (t, $\mathcal{J}_{2ax,1ax/3ax}$ = 9.6 Hz) suggested α-orientation of H-2, and hence the structure of compound **6** was deduced as 2*R*,8-dihydroxymenthol (**6**), formed by the C-2β hydroxylation of compound **2** (Scheme 1).

The EI-MS of compound **7** exhibited the $[M - CH_2OH]^+$ peak at m/z = 157. The HRFAB-MS (+ve) showed the $[M + H]^+$ ion at m/z = 189.1479, corresponding to the formula $C_{10}H_{21}O_3$, as a dihydroxy derivative of **1**. The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) was similar to that of **1**, with additional AB doublets at δ = 3.73/3.42 ppm ($\mathcal{J}_{AB}$ = 11.2 Hz) due to an oxygen-bearing CH_2. Moreover, a 3H singlet at δ = 1.20 ppm and the disappearance of H_3-9 suggested the vicinal



Scheme 1. Biotransformation of (+)-menthol by *M. phaseolina*.

hydroxylation at C-8 and C-9. The ^{13}C NMR spectrum of **7** lacked C-8 and C-9 signals, in comparison to **1**, and showed an additional quaternary carbon at δ = 76.9 ppm (C-8) and a methylene carbon at δ = 68.7 ppm (C-9). The position of the OH groups at C-8 and C-9 was further inferred on the basis of HMBC cross-peaks between C-10 methyl protons (δ = 1.20 ppm) and C-8 (δ = 76.9 ppm) and C-9 (δ = 68.7 ppm). The stereochemistry of C-8 OH was further inferred from the chemical shift (in $CDCl_3$) and by comparison with the reported ^{13}C NMR configuration at C-8 of an epimeric mixture of 8, 9-dihydroxymenthol (Yuasa & Yuasa 2004). C-10 in **7** appeared at δ = 23.3 ppm, which was in agreement with reported data for 8*S*,9-dihydroxymenthol. Thus the structure of compound **7** was deduced as 8*S*,9-dihydroxymenthol, which may be formed by the C-9 or C-7 hydroxylation of compound **2** or **5**, respectively (Scheme 1).

Compound **8** showed the $[M + H]^+$ ion at *m/z* = 189.1399 in HRFAB-MS (+ve), corresponding to the formula $C_{10}H_{21}O_3$. The ^1H NMR spectrum showed an additional downfield signal at δ = 3.10 ppm (dt, $J_{6ax,5eq}$ = 4.3 Hz, $J_{6ax,5ax/1ax}$ = 10.4 Hz), indicating the presence of a hydroxyl group at C-5 or C-6. Moreover, C-8 secondary methyls appeared as singlets at δ = 1.17 and 1.20 ppm, supporting a hydroxyl at C-8. The ^{13}C NMR spectrum of **8** showed two additional signals, one methine (δ = 76.3 ppm) and one quaternary carbon signal (δ = 75.1 ppm), along with the upfield shift of the C-7 signal (δ = 18.6 ppm). These observations further supported the position of the OH at C-6 and C-8. H-6 showed vicinal coupling with H-1 (δ = 1.48 ppm) and H_2-5

(δ = 1.81, 1.37 ppm) in the COSY 45° spectrum. The β-stereochemistry of the hydroxyl group was deduced by the multiplicity of the 6-αH signal (δ = 3.10 ppm, dt, $\mathcal{J}_{6ax,5eq}$ = 4.3 Hz, $\mathcal{J}_{6ax,5ax/1ax}$ = 10.4 Hz) and the NOESY correlations of H-6α with H_3-7 (δ = 0.91 ppm) and H-4α (δ = 1.42 ppm). Thus compound **8** was characterized as 6*R*,8-dihydroxymenthol, which may be formed by the monohydroxylation at C-8 or C-6β of compound **2** or **3**, respectively (Scheme 1).

The HRFAB-MS of metabolite **9** displayed an $[M + H]^+$ peak at *m/z* = 189.1411 corresponding to the formula $C_{10}H_{21}O_3$ and indicating the incorporation of two oxygen moieties in **1**. The ^{1}H NMR spectrum of compound **9** showed three methyl singlets at δ = 1.25 (H_3-7), 1.24 (H_3-9) and 1.23 (H_3-10) ppm. The ^{13}C NMR spectrum of **9** showed two quaternary carbon signals at δ = 71.5 and 75.6 ppm, while the disappearance of C-1 and C-8 methine carbons suggested that hydroxylation had taken place at C-1 and C-8. The HMBC spectrum of **9** showed the interactions of H_3-7 (δ = 1.25 ppm), H_2-2 (δ = 1.92, 1.40 ppm) and H_2-6 (δ = 1.61, 1.38 ppm) with C-1 (δ = 71.5 ppm), while H_3-9 (δ = 1.24 ppm) and H_3-10 (δ = 1.23 ppm) and H-4 (δ = 1.38 ppm) were correlated with C-8 (δ = 75.6 ppm). The NOESY spectrum showed the interaction of H_3-7α with H-4α, supporting a β-orientation of OH at C-1. Thus the structure of **9** was deduced as 1*R*,8-dihydroxymenthol, formed by C-1β or C-8 hydroxylation of compound **2** or **4**, respectively (Scheme 1).

The $[M + H]^+$ ion of compound **10** was found to be at *m/z* = 189.1438, corresponding to the formula $C_{10}H_{21}O_3$, in HRFAB MS (+ve). The ^{1}H NMR spectrum of **10** showed an additional doublet at δ = 3.47 ppm (2H, $\mathcal{J}$ = 6.2 Hz) and disappearance of the H_3-7 signal, which indicated oxidation of the C-7 methyl to C-7 CH_2OH. Second, a 6H singlet at δ = 1.21 ppm, due to the two methyls of an isopropyl moiety, indicated that another hydroxyl group was introduced at C-8. The ^{13}C NMR spectrum of **10** showed the disappearance of the C-8 methine and C-7 methyl carbon signals, in comparison to **1**, while oxygen-bearing quaternary and methylene carbons appeared at δ = 75.5 and 67.9 ppm, respectively. The position of the OH at C-7 and C-8 was further inferred by HMBC between H-4 (δ = 1.41 ppm) and H_6-9/10 (δ = 1.22 ppm) and with C-8 (δ = 75.5 ppm), while H-1 (δ = 1.51 ppm) showed cross-peaks with C-7 (δ = 67.9 ppm). The structure of compound **10** was deduced as 7,8-dihydroxymenthol, which may be formed by the C-7 hydroxylation of compound **2** (Scheme 1).

Time-course studies on compound **1** showed that the monohydroxylated derivatives, **2** and **4**, appeared after 3 days' incubation, while all transformation products **2**–**10** could be visualized by TLC together with residual compound **1** after 7 days. Further continuation of the experiment increased the yields of transformed products until day 12.

Conclusions

M. phaseolina performed regio- and stereocontrolled hydroxylations of **1** at C-1β, C-2β, C-5β, C-7, C-8 and C-9, which resulted in the production of nine hydroxylated products **2**–**10**. A hydroxyl group at C-8 was common in all dihydroxylated derivatives, indicating preferential oxidation at this position in **1** by *M. phaseolina*. The dihydroxylated derivatives, **6**–**10**, were found to be new compounds.

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