## Biotransformation of Terpenic Compounds by Fungi I. Metabolism of R-(+)-Pulegone

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Abstract: R-(+)-Pulegone 1 is converted by several fungal strains to new regioselectively hydroxylated compounds. Epoxidation of the double bond does not seem to be responsible for the main observed hydroxylation pattern.

R-(+)-Pulegone 1, a mintlike odor monoterpene ketone, constitutes the main component (up to 80-90%) of *Menta pulegium* essential oil (Pennyroyal oil) which is sometimes used in beverages and food for human consumption and occasionally in local herbal medicine as an abortifacient drug. The metabolism of pulegone<sup>1</sup> in animal has been investigated, and its hepatotoxicity has been related to the formation of chemically reactive metabolites (mainly menthofuran) derived from allylic oxidation of one of the methyl groups<sup>2-4</sup>. Several other compounds are formed *in vivo* in the rat<sup>5</sup>, deriving from the epoxidation of the double bond, further oxidation of the hydroxylated methyl group and/or direct hydroxylation of unactivated tertiary carbon of the ring.

We have investigated the biotransformation products of  $R_{+}$ -pulegone by fungal microorganisms<sup>6</sup>, with the hope to find regioselectively oxidized or hydroxylated derivatives to be used as asymmetric synthons, chiral auxiliaries or intermediates in organic synthesis.  $R_{+}$ -pulegone itself, which is in nature of very high optical purity, is a distinguished member of the "chiral pool" and has been currently used as a source for asymmetric C-methyl compounds<sup>7</sup>; a procedure for the large scale preparation of its S-enantiomer has been recently described<sup>8</sup> to make this compound available in both enantiomeric forms.

Most fungal strains tested (Table 1), grown in a usual liquid culture medium, were able to metabolize to some extent added R-(+)-pulegone in a concentration range of 0.1-0.5 g/litre; higher concentrations were generally toxic, excepted for one of the strains (*Aspergillus sp.*), isolated from mint leaves infusion, which was able to survive to concentrations up to 1.5 g/litre. Analysis of the incubation media revealed generally a complete metabolization of pulegone in 7-15 days, with the formation of a limited number of significant products which were detected and separated by GPC and TLC. The predominant product was generally 2 which was isolated and purified by solvent extraction and silica gel chromatography from a batch incubation<sup>9</sup> with *Aspergillus sp.* Compound 2 was identified by high resolution-mass spectrometry and <sup>1</sup>H and <sup>13</sup>C-NMR spectroscopy<sup>10</sup> as a 1-hydroxylated pulegone; moreover mild acidic dehydration of 2 (0.1 N HCl, 25°C) led to known piperitenone 3, identified again by NMR<sup>11</sup> and which was already present to some extent in the biotransformation products. The yield of 2 was about 20-30% (much lower than indicated by the GPC ratio of products) owing to losses of highly volatile pulegone during incubation. Other metabolites were constantly present in lower amounts (5% or less) and were tentatively identified as 4 (and its reduced derivative 5) and 6, from their <sup>1</sup>H- and <sup>13</sup>C-NMR data<sup>12</sup>. Acidic dehydration of 5 (0.1 M H2SO4, 80°C, 12 hours) regenerated R-(+)-pulegone.

	Incubation time(days)	1 (%) <sup>a</sup>	2 (%) <sup>a</sup>	3 (%) <sup>a</sup>	4 (%) <sup>a</sup>	5 (%) <sup>a</sup>
Aspergillus sp. <sup>b</sup>	7	2.5	58	10	4	6
Curvularia lunata (NRRL 2380)b	5	2	3	18	-	-
Rhizopus arrhizus (ATCC 11145) <sup>b</sup>	7	7	45	11	9	5
Sporotrichum exile (OM 1250) <sup>b</sup>	15	7	4.5	32	-	9
Mucor plumbeus (CBS 110-16) <sup>b</sup>	11	12	30	8	14	-
Mortierella isabellina (MMP 108) <sup>b</sup>	10	39	37	6	-	18

Table 1: Biotransformation of R-(+)-pulegone (0.5 g/litre) by various fungal strains

<sup>a</sup>% of total area of GPC peaks; DBwax capillary column (0.25mm I.D. x 30 m) run at 125 to 180°C (5°C/min.); carrier gas: helium (1.5 bar). <sup>b</sup> ATCC: American Type Culture Collection (Rockville, Maryland, USA); CBS: Centraalbureau voor Schimmelcultures (Baam, Netherland); MMP: Mycothèque du Muséum d'Histoire Naturelle (Paris, France); NRRL: Northern Utilization Research Branch, US Dept Agriculture (Peoria, III.,USA); QM: Quartermaster Culture Collection, Univ. Massachusetts, Amherst, Mass., USA); others: locally isolated strains.

Quite interestingly, incubation of a crude Pennyroyal oil from Morocco afforded very similar results. A tentative metabolic pathway for explaining the formation of such products by *Aspergillus sp.* is given in Fig.1. Formation of major compound 2 may be explained by direct hydroxylation on a tertiary position as previously described in rat metabolism of R-(+)-pulegone<sup>5</sup>. Its dehydration to piperitenone 3, even in the incubation conditions, or during isolation or derivatization reactions precluded any tentative to determine its optical purity and absolute configuration.



Figure 1: Metabolic Pathways for the Formation of Main Products Found in the Biotransformation of R-(+)-Pulegone by Aspergillus sp.

The formation of the new hydroxylated product 4 (and derivatives 5 and 6) is difficult to explain, even as the result of an intermediate epoxidation of the double bond: one has to suppose the formation of the adjacent diol (which is known in the metabolism of other open chain monoterpenes<sup>13,14</sup>), followed by a dehydration reaction involving a hydroxy group in an unfavourable position ( $\alpha$ - to the carbonyl). Other possible hypotheses would be: i) a concerted opening of the epoxide involving hydrogen elimination in position -5, with a driving force deriving from the formation of an endocyclic conjugated double bond, or ii) a direct hydroxylation of position -5 followed by an allylic alcohol rearrangement (as illustrated in Fig.1 for the formation of 4). It is to be noted that no epoxide could be detected at any time in the products of the biotransformation.

In order to elucidate this point, a mixture of diastereoisomeric epoxides 7 and 8 (Fig.2) was prepared<sup>15</sup> and incubated with Aspergillus sp. in conditions similar to the previous ones: only the trans-(1R,4R)-epoxide 7 was (slowly) metabolized affording a mixture of products, among which a diol 9 was identified<sup>5,16</sup> as a major product, but without any trace of the hydroxy metabolites 4 or 5 precedently formed from pulegone. The formation of the hydroxy compound 4 does not result either from a mild acidic treatment of the epoxide mixture, which affords the diastereoisomeric diols mixture 9, while a more prolonged treatment leads essentially to alcohol  $10^{17}$ , probably through dehydration of the intermediate diol. Such results seem to rule out definitively any pathway involving intermediate epoxide formation in the bioconversion of pulegone into the unsaturated alcohol derivative 4.



Figure 2: Reactions of R-(+)-Pulegone Epoxides: *i*, H2O2 / MeOH; *ii*, 0.1 M H2SO4, 4°C; *iii*, 0.1N HCl, 70°C.

These data emphazise the ability of this Aspergillus strain to effect direct hydroxylation reactions rather than the classical epoxide formation and metabolism. Its use as a general hydroxylation tool for other cyclic substituted keto chemicals or natural products is currently in progress.

## **References and notes:**

- 1- A large number of biotransformations of terpenoid compounds, including mono-, sesqui- and diterpenes have been investigated in animals, or using plant cells or microorganisms cultures; for detailed reviews of such data, see Krasnobajew, V. . In "Biotechnology, a Comprehensive Treatise", vol.6a: Biotransformations; Rehm, H.-J.; Reed, G. Eds.; Verlag Chemie; Weinheim, 1984; pp.31-77; Santhanakrishnan, T.S. Tetrahedron, 1984, 40, 3597-3609; Lamare, V.; Furstoss, R. Tetrahedron, 1990, 46, 4109-4132.
- 2- Gordon, W. P.; Forte, A. J.; McMurtry, R. J.; Gal, J.; Nelson, S. D. Toxicol.Appl.Pharmacol. 1982, 65, 413-424..
- 3- Gordon, W. P.; Huitric, A. C.; Seth, C. L.; McClanahan, R. H.; Nelson, S. D. Drug Metabolism and Disposition . 1987. 15, 589-594.

- 4- McClanahan, R. H.; Thomassen, D.; Slattery, J. T.; Nelson, S. D. Chem.Res.Toxicol. 1989, 2, 349-355.
- 5- Moorthy, B.; Madyastha, P.; Madyastha, K. M. Xenobiotica 1989, 19, 217-224.
- 6- Lassak, E.V., Pinhey, J.T.; Ralph, B.J.; Sheldon, T.; Simes, J.J.H. Aust. J. Chem. 1973, 26, 845-854.
- 7- Among the first numerous examples of use of R-(+)-pulegone as a source of asymmetric synthons see typically: Godchot, M.; Cauquil, G.; Calas, R. Bull Soc Chim. 1939, 6, 1353-1358; Eisenbraun, E.J.; McElvain, S.M. J.Am.Chem.Soc. 1955, 77, 3383-3384; for a complete set of more recent references, see Merck-Schuchardt Infos 84-12.
- 8- Bushmann, H.; Scharf, H. D. Synthesis 1988, 827-829.
- 9- Aspergillus sp. was grown from freshly obtained spores during 3 days at 27°C in 250 ml orbitally shaken conical flasks containing 100 ml of the following culture medium : NaNO3, 2g; K2HPO4, 2g; KH2PO4, 1g; MgSO4, 7H2O, 0.5 g; KCl, 0.5 g; FeSO4, 7H2O, 0.02 g; corn steep liquor, 10 g and glucose, 30 g, in 1 litre of distilled water. R-(+)-pulegone (1 g/litre) was added and incubation was continued for 5 days. The filtered incubation medium was saturated with NaCl then extracted repeatedly with EtOAc; the organic extract was chromatographed on silica gel (Merck H60) with mixtures of cyclohexane-EtOAc as solvent. Further purification of fractions for analysis was effected by preparative TLC.
- 10- 2, [α]<sub>D</sub><sup>20</sup>= -14° (c 1, CHCl<sub>3</sub>); HRMS for C<sub>10</sub>H<sub>16</sub>0<sub>2</sub>, calc.168.114481, found 168.115666; MS, m/z (% relative abundance): 168 (5), 156 (30), 150 (12), 135 (12), 125 (23), 107 (72), 97 (85), 85 (100), 67 (40). <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 250 MHz), δ ppm: 3.04 (br.s., OH), 2.40 (m, 2 CH<sub>2</sub>), 1.88 and 1.70 (2s, =C-CH<sub>3</sub>), 1.80 (m, CH<sub>2</sub>), 1.20 (s, CH<sub>3</sub>-7). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 62.9 MHz) δ ppm: 202.3 (CO), 143.2 (C-8), 130.4 (C-4), 71.6 (C-1), 55.8 (C-2), 36.9 (C-6), 29.3 (CH<sub>3</sub>-7), 24.9 (C-5), 23.0 and 22.3 (CH<sub>3</sub>-9 and -10). No splitting of any <sup>1</sup>H-NMR signal could be observed upon increasing additions of a chiral shift reagent [Eu(hfc)<sub>3</sub>] to 2 in CDCl<sub>3</sub> solution.
- 11- 3, <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 250 MHz), δ ppm: 5.86 (q, J<sub>4,7</sub>= 1.5 Hz, H-2), 2.63 and 2.26 (2t,J= 6.5 Hz, 2 CH<sub>2</sub>), 2.07 (s, CH<sub>3</sub>-7), 1.90 and 1.83 (2s, CH<sub>3</sub>-9 and -10). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 62.9 MHz) δppm: 196.7 (CO), 167.3 (C-8), 129.0 (C-2), 31.9 (C-4), 27.9 (C-5), 23.8, 22.9 and 22.50 (CH<sub>3</sub>-7, -9 and -10).
- 12- 4,  $[\alpha]_D^{20}$ = -49°(c 0.4, CHCl<sub>3</sub>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 250 MHz),  $\delta$  ppm: 6.86 (dd, J<sub>5,6</sub>= J<sub>5,6</sub>= 6 Hz, H-5), 4.38 (br.s, OH), 2.46 and 2.12 (2m, 2 CH<sub>2</sub>+CH), 1.36 (s, CH<sub>3</sub>-9 and 10), 1.02 (d, J= 6 Hz, CH<sub>3</sub>-7). 5, <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 250 MHz),  $\delta$  ppm: 3.95 (br.s, OH), 2.32 and 2.11 (2m, H-2 and H-6), 1.95,1.86, 1.50 and 1.35 (4m, 5 CH), 1.19 and 1.18 (2s, CH<sub>3</sub>-9 and -10), 1.00 (d, J= 6.5 Hz, CH<sub>3</sub>-7). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 62.9 MHz)  $\delta$  ppm: 215.1 (CO), 71.3 (C-8), 58.8 (C-4), 51.5 (C-2), 35.5 (C-1), 33.9 (C-6), 28.7 (C-5), 28.7 and 25.6 (CH<sub>3</sub>-9 and -10), 22.2 (CH<sub>3</sub>-7).

6 (see ref.6), <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 250 MHz),  $\delta$  ppm: 5.85 (br.s, =CH), 2.07 (m, H-4), 1.95 (s, CH<sub>3</sub>-7), 1.19 and 1.18 (2s, CH<sub>3</sub>-9 and -10).

- 13- Abraham, W.R.; Hoffman, H.M.R.; Kieslich, K.; Reng, G. and Stumpf, B., in *Enzymes in organic synthesis, CIBA Foundation Symposium n°111*, Porter, R.; Clark. S. Eds; Pitman; London, 1985; pp. 146-160.
- 14- Yamazaki, Y.; Hayashi, Y.; Hori, N.; Mikami, Y. Agric.Biol.Chem. 1988, 52, 2921-2922
- 15- Katsuhara, J. J.Org.Chem. 1967, 32, 797-799. For a conformational study of cis- and trans-epoxides, see Feeley, T.M.; Hargreaves, M.K. J.Chem.Soc.(C), 1970, 1745-1750. 7. <sup>1</sup>H-NMR (CDCl3, 250 MHz), δ ppm: 2.38 (br.s, w1/2= 6 Hz, CH), 2.16 ,1.95, 1.80 (3m, CH), 1.38, 1.17 (2s, CH3-9 and -10), 1.02 (d, J= 6.5 Hz, CH3-7); 8: 2.55 (dt, J= 13 and 3 Hz, CH), 2.37 (br.s, CH), 1.95, 1.85 (2 m, CH), 1.38, 1.16 (2 s, CH3-9 and -10), 1.01 (d, J= 7 Hz, CH3-7). 7+ 8, <sup>13</sup>C-NMR (CDCl3, 62.9 MHz), δ ppm: 207.4 and 206.3 (CO), 70.2, 70.1, 63.4 and 63.1 (C-4 and -8), 51.4 and 49.5 (C-2), 33.9 and 30.7 (C-1), 33.0, 30.2, 29.9, 26.3 (C-5 and C-6), 22.0, 20.0, 19.7, 19.6, 19.4, 19.0 (CH<sub>3</sub>-7, -9, -10).
- 16- 9, <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 250 MHz),  $\delta$  ppm: 3.80 (br.s, OH), 2.54 (dd, J<sub>2,2'</sub> = 14 Hz, J<sub>2,1</sub> = 12 Hz, H-2), 2.22 (dd, J<sub>2',2</sub> = 14 Hz, J<sub>2',1</sub> = 4 Hz, H-2), 1.27 and 1.12 (2 s, CH<sub>3</sub>-9 and -10), 0.95 (d, J = 6.5 Hz, CH<sub>3</sub>-7). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 62.9 MHz),  $\delta$  ppm: 214.1 (CO), 78.1, 74.9 (C-4 and C-8), 47.2 (C-2), 33.5 (C-1), 32.6, 28.8 (C-5 and C-6), 25.2, 23.5, 21.8 (CH<sub>3</sub>-7, -9 and -10).
- 17- 10, <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 250 MHz), δ ppm: 5.10 and 5.05 (2 d, =CH<sub>2</sub>), 4.11 and 4.03 (br.s, OH), 2.71 and 2.55 (2 dd, H-2), 1.66 (s, =C-CH<sub>3</sub>), 1.01 and 0.95 (2 d, CH<sub>3</sub>-7).

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