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Regio- and Stereoselective Aspects in the Oxidation of (*R*) and (*S*) 4a-Methyl-(4,4a,5,6,7,8)-hexahydro-2(3H)naphthalenones in Living Rats

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Abstract : The bioconversion of (R)-1 and (S)-1 in living rats was studied. The urinary hydroxylated metabolites obtained were identified by using GC-MS and ^{1}H NMR spectroscopy. Oxidation of octalenones (R)-1 and (S)-1 in living rats took place at three sites: positions 6,7 and 8. However, for a given site, this oxidation was highly stereoselective: only one hydroxylated diastereomer was always obtained. With the exception of metabolite 6, the oxidation always occurred in the syn position relatively to the angular methyl group. These results are significantly different from those observed, using "alternative methods", such as microsomal and microbial biodegradations. These data show that from a stereochemical and a regiochemical viewpoint,"alternative methods" need to be validated by controls in living animals.

INTRODUCTION

Many methods have been recommended as alternatives to experiments in living animals for studies of xenobiotic metabolism: hepatic microsomes, freshly isolated hepatocytes, isolated perfused organs, tissue homogenates, hepatoma cells in culture, isolated purified enzymes, reconstructed model systems etc., but *in vivo* findings cannot be used in a predictive manner without a careful validation.

Microorganism such as *Rhizopus arrhizus* have been known as capable of stereoselective oxidation of ketosteroids[1], but with (*S*)-4a-methylhexahydronaphthalenone [(*S*)-**1**], no stereoselectivity was obtained[2]. Recently, the regio- and stereoselective oxidation of substituted optically active octalenones by selected fungal strains, including *Mucor plumbeus* MMP 430, has been shown to afford various optically pure hydroxylated metabolites[3,4]. However, to our knowledge, the direct oxidation of (*R*)-**1** and (*S*)-**1** in living animals has not been examined so far. In this paper we report on the bioconversion of both enantiomers of octalenone **1** in living rats, by investigating the urinary metabolites.

RESULTS AND DISCUSSION

The two enantiomers (*R*)-**1** and (*S*)-**1** were administered to lots of female rats at the daily dose of 20 mg/kg. After extraction from urines, the metabolites were purified by using various chromatographic systems and then identified by ^{1}H NMR and EI-MS spectroscopy. The structures of the metabolites are displayed in Schemes 1 and 2, and their relative percentages are given in Table 1.

(*R*)-**1**	**2**	**3**	**4**	**5**	
	2.2 ± 0.3	1.6 ± 0.2	15.2 ± 1.5	4.7 ± 0.6	
(*S*)-**1**	*ent*-**2**	*ent*-**3**	**6**	*ent*-**5**	**7**
	2.3 ± 0.1	2.0 ± 0.1	4.2 ± 0.3	traces	traces

Table1: Percentages of hydroxylated urinary metabolites (as compared to the total amount of starting material) obtained after administration of (*R*)-**1** and (*S*)-**1** to living rats.

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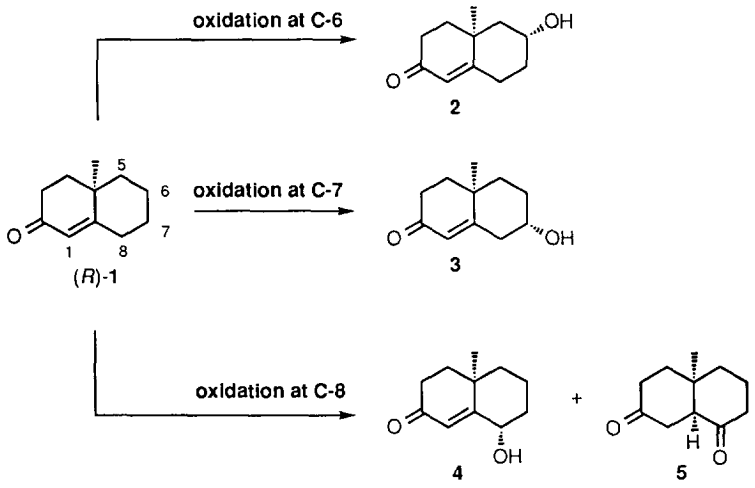
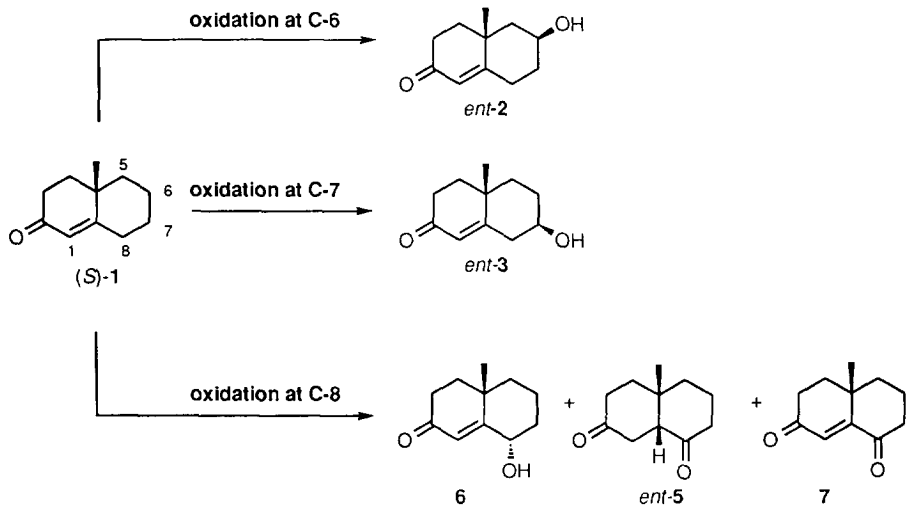


Scheme 1: Metabolic pathway of (*R*)-**1** in Rat.



Scheme 2: Metabolic pathway of (*S*)-**1** in Rat.

These experimental outcomes deserve several comments:

- In both cases no significant amount of octalenones was recovered.
- Positions 6, 7 and 8 of the two enantiomeric octalenones were oxidized, but when the percentages of oxidation at the position 6 and 7 are similar, the oxidation at position 8 is much more important for enantiomer (*R*)-**1** than for (*S*)-**1**. In the case of (*R*)-**1**, a substantial amount of diketone **5** was formed, while only a trace amount of (*ent*)-**5** was obtained with (*S*)-**1**.

• For the metabolites of (*R*)-**1** or (*S*)-**1** oxidized in position 6 or 7, the entering hydroxyl function is always in the *syn* position relatively to the angular methyl group. In contrast for the 8-hydroxylated metabolites, the oxidation took place in the *syn* position relatively to the methyl group for (*R*)-**1**, and in the *anti* position for (*S*)-**1**.

• The ring junction in both metabolites **5** and *ent*-**5** is *cis* (thermodynamically less stable configuration).[3,4] These results can be compare with other methods of bioconversion of (*R*)-**1** and (*S*)-**1**:

• Microsomal oxidation: a japanese group[5] has investigated the bioconversion of (*S*)-**1** by using rat microsomes. The only diastereomeric 8-hydroxylated metabolites were obtained, in the *cis/trans* ratio of 14/1.

• Microbial hydroxylation: Holland *et al* [1,2] studied the bioconversion of (*S*)-**1** by *Rhizopus arrhizus*. Similar results as those described by microsomal oxidation were obtained. A more complete study was made by Azerad *et al*[3,4], using (*R*)-**1** and (*S*)-**1** and a set of various fungal strains: *Absidia glauca, Beauvaria bassiana, Cunninghamella echinulata, Curvalaria lunata, Cylindrocarpon radicicola, Mucor aromaticus, Mucor jansenii, Mucor plumbeus, Mucor racemosus, Mucor rouxii.* In these cases, no 7-hydroxylated metabolites were obtained. In both series, 6-hydroxylated and 8-hydroxylated metabolites exhibited the *syn* relationship between the entering hydroxyl function and the angular methyl group.

CONCLUSION

Oxidation of octalenones (*R*)-**1** and (*S*)-**1** in living rats took place in three sites: positions 6,7 and 8. However for a given site, this oxidation is highly stereoselective: only one hydroxylated diastereomer was always obtained. With the exception of metabolite **6**, the oxidation always occurred in the *syn* position relatively to the angular methyl group. These results are significantly different from those observed, using "alternative methods", such as microsomal and microbial biooxidations. These data show that, from a regio- and stereochemical viewpoint, alternative methods need to be validated by control in living animals.

EXPERIMENTAL PART

General. ^{1}H NMR spectra were recorded at 200 MHz on a Bruker AC 200 and at 400 MHz on a Bruker ARX 400 spectrometer. ^{13}C NMR spectra were recorded at 50 MHz on a Bruker AC 200 spectrometer. Recognition of methyl, methylene, methine and quaternary carbon nuclei in ^{13}C NMR spectra rests on the *J*-modulated spin-echo sequence. Elemental analyses were obtained from the Service de microanalyse, Centre d'Etudes Pharmaceutiques, Châtenay-Malabry, France. Analytical thin-layer chromatography was performed on Merck silica gel 60 F_{254} glass precoated plates (0.25 mm layer). All liquid chromatography separations were performed using Merck silica gel 60 (230-400 mesh ASTM). A Hewlett Packard 5890 gas chromatograph equipped with a capillary column was used for the GC analyses. The fused silica column (25 m x 0.2 mm ID x 0.11 µm film thickness) was coated with crosslinked 5 % phenylmethyl silicon gum. The carrier gas was helium at an inlet pressure of 62 kPa. Mass spectra were obtained on a GC-MS system. The mass spectrometer (Hewlett Packard 5970 MSD) was operating in electron impact mode (70 eV) and directly interfaced with the gas chromatograph apparatus. The specific rotations were determined at 589 nm in 1 dm-cell with a Schmidt-Haensch polarimeter (Polartronic E).

Starting materials. (*S*)-**1** and (*R*)-**1** 4a-methyl-4,4a, 5,6,7,8-hexahydro-2(3H)naphthalenones were prepared as previously described.[6] Retention times in GC: 18.3 and 18.7 min respectively; $[\alpha]_D^{21}$ = +212 (c 1.0, EtOH) and $[\alpha]_D^{21}$= -210 (c 1.0, EtOH) respectively (ee ≥ 97 %).

Animals. Sprague Dawley female rats were placed in Pajon metabolism cages (3 rats per cage). They had free access to food and water.

Formulation . The total amount of octalenone was solubilized in 20 ml ethanol. The ethanolic solution was mixed 10 minutes with a suspension of Carboxymethylcellulose (CMC) (12 *M*) 0.212 % in water. The volume of CMC was calculated to give 1 ml of suspension per day and per rat.

Administration. The volume of octalenone suspension was adapted to give a dose equivalent to 20 mg/kg/day and daily administered directly in the stomach with a curved cannula 60/100 (Carrieri). Data concerning the experiments are summarized in Table 2.

	Experiment	Drug	Treatment duration	Animals	
		Total administration (g)	(day)	Number	Total weight (kg)
(*R*)-**1**	1	2.58	10	44	12.90
	2	2.35	10	44	11.77
	3	2.56	10	40	10.67
(*S*)-**1**	4	2.92	12	42	12.19
	5	2.79	12	42	11.64
	6	3.10	16	40	9.67

Table 2: Administration protocol of (*R*)-**1** and (*S*)-**1** to rats.

Collection of urines. Urines were collected and frozen (-18 °C) each day during the administration, and two days after the end of the treatment.

Extraction of metabolites from urines of treated rats. At the end of the experiment, pooled frozen urines were allowed to warm up at room temperature and filtered. The samples were divided into 1 l -aliquots. Each aliquot was extracted three times at pH of urine (pH 9) with ethyl acetate (1 l). The extraction was then repeated at three different pH : 7, 5, 1, after acidification with 6 *N* HCl. Combined organic solutions were dried over anhydrous sodium sulfate and evaporated to dryness under vacuum at room temperature.

Isolation and identification of metabolites. Residues were taken up with ethyl acetate and an aliquot was analyzed by GC-MS spectrometry. After chromatography over silica gel (ethyl acetate-cyclohexane 1:1), the purified metabolites were characterized by ^{1}H NMR and ^{13}C NMR spectroscopy , mass spectrometry and polarimetry.

(4aS, 6R)-cis-4a-methyl-6-hydroxy-4,4a,5,6,7,8-hexahydro-2(3H) naphthalenone (**2**).
Mp = 124-125 °C; $[\alpha]_D^{21}$ = -205 (c 0.9, $CHCl_3$); GC retention time: 24.2 min.; MS, 180(44) M^+, 162(38), 152(36), 134(38), 123(56), 108(100), 91 (89), 73(63), 67(15), 55(31); ^1H and ^{13}C NMR spectra were in complete agreement with those previously published[4] .

(4aR, 6S)-cis-4a-methyl-6-hydroxy-4,4a,5,6,7,8-hexahydro-2(3H) naphthalenone (***ent*-2**).
Mp = 122-125 °C; $[\alpha]_D^{21}$ = +210 (c 0.85, $CHCl_3$); GC retention time: 24.7 min.; ^1H and ^{13}C NMR spectra were in complete agreement with those previously published[4] .

(4aR, 7S)-4a-methyl-7-hydroxy-4,4a,5,6,7,8-hexahydro-2(3H) naphtalenone (**3**).
Amorphous solid; $[\alpha]_D^{21}$ = -83 (c 1.0, MeOH); Anal. $C_{11}H_{16}O_2$ (180.25), calc.%: C, 73.29, H, 8.94; found %: C, 73.48, H, 8.99; GC retention time: 24.1 min.; MS, 180(26) $M^{+\cdot}$,162(12), 152(20), 134(25), 124(100), 109(59), 91(65), 79(49), 67(26), 55(31); ^1H NMR (400 MHz,$CDCl_3$) δ 1.14 (s, 3H), 1.24 (ddd, 2H, J = 12.4 Hz, J = 12.4 Hz, J = 4,7 Hz), 1.26-2.10 (m, 4H), 2.20-2.40 (m, 4H), 2.60 (broad s, 1H), 3.60 (dddd, J = 11.0 Hz, J = 10.8 Hz, J = 5.0 Hz, J = 4.5 Hz, 1H), 5.70 (d, J = 1.2 Hz, 1H); ^{13}C NMR (50 MHz,$CDCl_3$) δ: 198.1 (C), 166.3 (CH), 125.8 (CH), 70.6 (CH), 42.2 (CH_2), 38.7 (CH_2), 37.4 (CH_2), 34.8 (C), 34.1 (CH_2), 31.3 (CH_2), 21.6 (CH_3).

(4aS, 7R)-4a-methyl-7-hydroxy-4,4a,5,6,7,8-hexahydro-2(3H) naphtalenone (***ent*-3**).
Amorphous solid; $[\alpha]_D^{21}$ = +85 (c 0.95, MeOH); Anal. $C_{11}H_{16}O_2$ (180.25), calc.%: C, 73.29, H, 8.94; found %: C, 72.93, H, 9.15; GC retention time: 24.3 min.; ^1H and ^{13}C NMR spectra were identical to those observed for **3** (*vide supra*).

(4aR, 8S)-4a-methyl-8- hydroxy-4,4a,5,6,7,8-hexahydro-2(3H) naphthalenone (**4**).
Mp = 66-68 °C; $[\alpha]_D^{21}$ = -92 (c 1.02, $CHCl_3$); GC retention time: 22.6 min.; MS, 180(94)M^+, 165(100), 152(28), 151(26), 147(18), 137(53), 124(99), 109(91), 91(97), 79(92), 67(76), 55(94); ^1H and ^{13}C NMR spectra were in complete agreement with those previously published[4] .

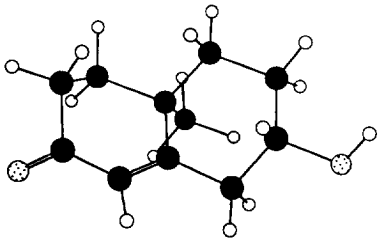
Cis(R)-4a-methyl-3,4,4a,5,6,8a-hexahydro-(2H,8H) naphthalene-1,7-dione (**5**).
GC retention time: 21.9 min.; MS, 180(44) $M^{+\cdot}$, 165(8), 151(100), 137(27), 123(67), 110(15), 97(26), 82(38), 67(48), 55(69); ^1H and ^{13}C NMR spectra were in complete agreement with those previously published[4,7] .

Cis(S)-4a-methyl-3,4,4a,5,6,8a-hexahydro-(2H,8H) naphthalene-1,7-dione (***ent*-5**).
GC retention time: 22.3 min.; ^1H and ^{13}C NMR spectra were in complete agreement with those previously published[4,7] .

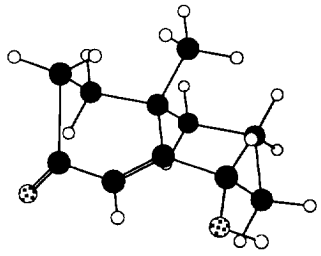
(4aS, 8S)-4a-methyl-8- hydroxy-4,4a,5,6,7,8-hexahydro-2(3H) naphthalenone (**6**)[8].
Oil; Bp (0.1 Torr) = 105-115 °C; $[\alpha]_D^{21}$ = +138 (c 3.95, $CHCl_3$); Anal. $C_{11}H_{16}O_2$ (180.25), calc.%: C, 73.29, H, 8.94; found %: C, 73.52, H, 8.75; GC retention time: 25.6 min; MS, 180(35) M+., 16(14), 152(12), 151(16), 147(7), 137(29), 124(87), 109(100), 95(52), 79(43), 67(67), 55(38); ^1H NMR (400 MHz, $CDCl_3$) δ 1.20 (s, 3H), 1.25-2.55 (m, 10H), 2.70 (broad s., 1H), 4.30 (ddd, J = 12.1 Hz, J = 5.6 Hz, J = 1.8 Hz, 1H), 6.15 (d, J = 1.8 Hz, 1H); ^{13}C NMR (50 MHz,$CDCl_3$) δ: 200.1 (C), 171.6 (C), 119.0 (CH), 68.6 (CH), 40.9 (CH2), 38.1 (CH_2), 36.4 (CH2 and 1C), 33.6 (CH2), 22.7 (CH3), 19.9 (CH2).

(S)-4a-methyl-3,4,4a,5-tetrahydro(2H,6H)naphthalene-1,7-dione (**7**).
GC retention time: 24.7 min.; MS, 178(82) $M^{+\cdot}$, 163(37), 150(16), 135(61), 121(89), 107(100), 91(78), 79(87), 77(74), 65(34), 55(43); ^1H NMR spectrum was in complete agreement with the published one.[8]

Note: Molecular modeling of the flexible bicyclic enone system, using PC model, indicated that both compounds ***3*** *and* ***6*** *exhibit a chair-like B-ring in their energy-minimized conformation. Furthermore the calculated coupling constants for* H_7 *in* ***3*** *and* H_8 *in* ***6****, consistent with axial protons, are in complete agreement with those experimentally observed.*



Molecular modeling (PC model) for **3**



Molecular modeling (PC model) for **6**

Coupling constants for H_7	
Observed	Calculated
J = 11.0 Hz	J = 11.1 Hz
J = 10.8 Hz	J = 10.8 Hz
J = 5.0 Hz	J = 5.1 Hz
J = 4.5 Hz	J = 4.8 Hz

Coupling constants for H_8	
Observed	Calculated
J = 12.1 Hz	J = 11.2 Hz
J = 5.6 Hz	J = 5.2 Hz
J = 1.8 Hz	not calculated

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