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Microbial Hydroxylation and Functionalization of Hydrindenones

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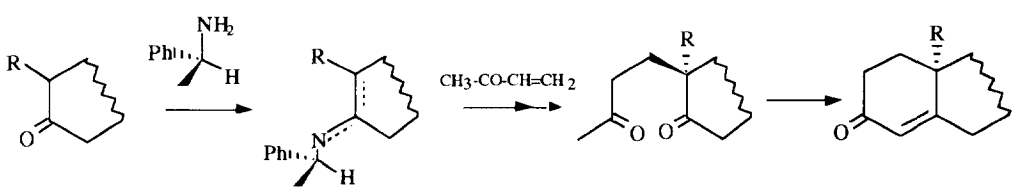
Abstract: The regio- and stereoselective hydroxylation of enantiomeric 4a-methyl hydrindenones by selected fungal strains has been shown to afford several enantiomerically pure derivatives, hydroxylated in the B-ring. The potential of this method for the preparation of hydroxylated chiral synthons is evaluated and discussed.

INTRODUCTION

Efficient deracemizing alkylations of 2-substituted cyclic ketones leading to optically active functionalized 2,2-disubstituted cyclanones, through diastereomeric imine formation, have been described and thoroughly investigated [1-8]. Such carbonyl compounds, bearing an stereogenic α-quaternary center, are obtained in high chemical yield and enantiomeric excess. They are easily converted to enantiomeric bicyclic enones [9] which may constitute useful building blocks for the elaboration of synthetic strategies in the field of steroids, terpenes, and related molecules [7].



However, the functionalization at selected carbons of these chiral synthons, necessary for further elaboration, is not easily achieved by chemical methods and remains a difficult challenge, particularly in the hydrindenone series. In fact, most of the published methods use as a starting material the corresponding racemic or optically active 2,5-dione (analogous to the Wieland-Miescher ketone), in which a second carbonyl group in the B-ring can be further transformed [10-13]: for example, the microbial reduction of the 5-keto group has been used to produce stereoselectively 5-hydroxylated derivatives [14]. On the contrary, the direct microbial hydroxylation of one of the methylenic groups of the rings, though frequently observed in the practice of steroid or diterpene biotransformations, has been only marginally explored in this respect [15]. We have recently described some results of the biotransformation of 4a-methyl octalone (and various substituted derivatives) enantiomers by several fungal strains, as well as the potential of such reactions in the production of regio- and stereoselectively hydroxylated derivatives [16,17]. We report in the present paper the extension of these results to 4a-methyl hydrindenones **1**, also available as pure enantiomers through the previously described alkylation method [1].

RESULTS AND DISCUSSION

M.plumbeus CBS 110-16, which was previously used for the hydroxylation of 4a-methyl octalones [16], was also found, in a preliminary screening, as an active strain for the hydroxylation of both enantiomers of hydrindenone **1**. However the disappearance of the hydrindenone substrates was slower and prolonged incubation was necessary to obtain high conversion yields. Several products were detected by TLC and a semi quantitative assessment of metabolite yields was obtained by GC-mass analysis of crude organic extracts (Table 1). In 4 days-incubations, when the *R*- or *S*-substrate conversion was limited to about 60%, one or two main hydroxylation products **A** and **B** were observed; in longer (7-10 days) incubations of *S*-**1**, a new hydroxylated product was additionally formed in significant amounts, among other minor products.

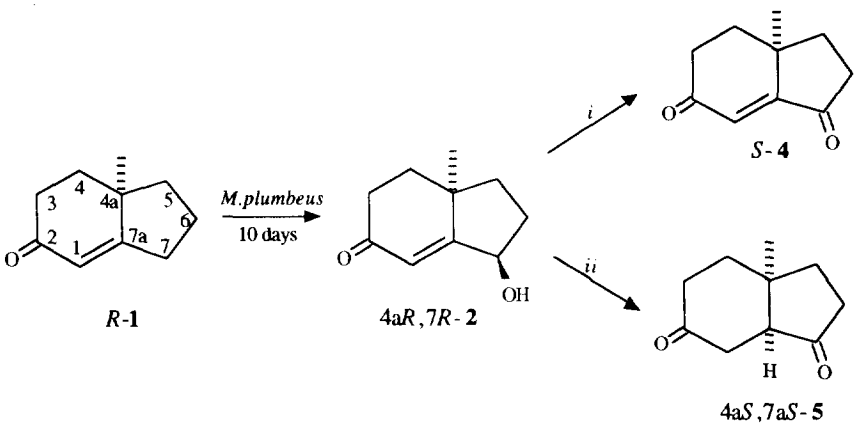
Table 1: Formation of Hydroxylated Products from S-1 or R-1 Added (0.5 g L^{-1}) to Grown Cultures of Various Fungal Strains

Strains[a]	Incubation time (days)	% products obtained[b] from *R*-1				% products obtained[b] from *S*-1			
		R-1	A	B	others	*S*-1	A	B	others
Mucor plumbeus (CBS 110-16)	2	55	29	6	6	52	11	27	4
	4	36	47	3	9	39	13	28	8
	7	19	62	1	11[c]	26	21	25	16[d]
	10	15	63	1	11[c]	27	18	23	15[d]
Beauveria bassiana (ATCC 7159)	2	53	12	17	11.5	54	13	17	10
	4	24	16	15	39	25	22	6	36[d]
	7	17	14	2	56[c]	15	18	0.5	48[d]
	10	12	14	2	52[c]	11	18	0.5	47[d]
Curvularia lunata (NRRL 2380)	2	70	11	15	1	71	12	12	1.5
	4	52	23	8	7	33	22	12	9
	7	20	27	-	26[c,d]	8	18	3	28[d]
	10	12	36	-	29[c,d]	5	22	3	31[d]

a) Origin of strains: ATCC, American Type Culture Collection (Rockville, Maryland, USA); CBS, Centraalbureau voor Schimmelcultures (Baarn, Netherlands); NRRL, Northern Utilization Research (Peoria, Illinois, USA). b) % area of the corresponding chromatographic peaks (DBwax capillary column; 200°C; retention times: **1**, 3.2 min; **2** (=**A**), 18.5 min; **3** (=**B**), 14.5 min; **4**, 7.5 min; **5**, 5.5 min; **6**, 8.5 min). c) mainly **5**. d) mainly **6**.

In a preparative 10 days-incubation of *R*-**1**, directly added to the culture medium (0.5 g L^{-1}), about 10% of the substrate was recovered unchanged, and the major hydroxylated product **A** (47%) was isolated from the incubation filtrate (Scheme 1). A (4a*R*,7*R*)-*trans*-7-hydroxy structure **2** was deduced from chemical evidence, from GC/MS data, and from a detailed ^{1}H and ^{13}C NMR study. The allylic hydroxylation product **2** was not completely stable: kept dry in air for a few weeks, it partially oxidized to the diketone ; the same diketone could be quantitatively obtained by Jones oxidation. In addition, an acidic treatment of **2** afforded a diketo saturated *cis*-rearrangement product **5**, as previously known for the corresponding 8-hydroxylated derivatives of 4a-methyl octalone [16,18]. The ^{1}H NMR signal of the *CH*OH group in compound **2** was located at 4.80 ppm, a high chemical shift in agreement with an allylic position of the OH group [19]. The complex pattern observed for the H-7 signal was elucidated by 2D ^{1}H-^{1}H COSY, selective homodecoupling experiments, and simulated spectra, which revealed a long range coupling (J = 1.5 Hz) between the H-7 and H-1 signals. The *trans* position of the hydroxyl group in **2**, suggested by the nearly identical location of the methyl group signal, compared to that of **1**, was confirmed by nOe experiments (Figure 1) where an effect was observed between H-7 and the 4a-methyl, when the latter was saturated (nOe's with H-3ax and H-6β were observed jointly). A nOe between H-1 and H-7 signals was additionally observed confirming the 7-position of the hydroxyl group.

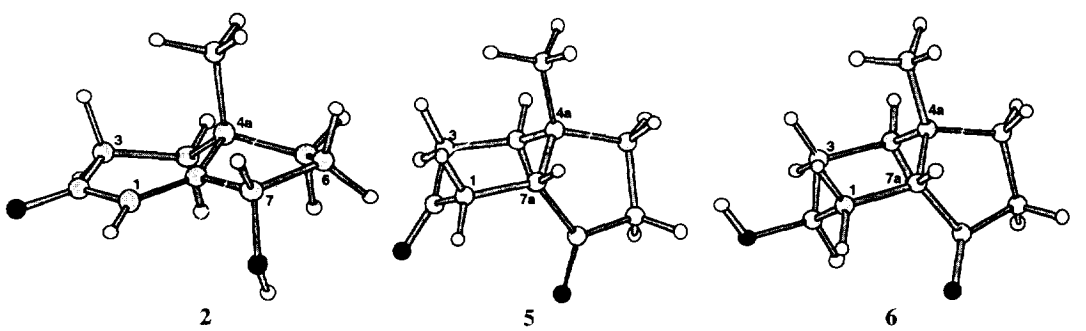
The *cis*-configuration of compound **5** was established through complete assignments of ^{1}H and ^{13}C NMR resonances, by 1D and 2D experiments, PFG-phase sensitive DQFCOSY, and ^{1}H-^{13}C direct and long range correlations (PFG-HMQC and PFG-HMBC). The H-7a signal was unambiguously assigned as the only CH



R-**1** 4a*R*,7*R*-**2** *S*-**4** 4a*S*,7a*S*-**5**

Scheme 1: i) Air oxidation, or CrO_3, H_2SO_4/acetone; *ii*) HBr /Et_2O

group in the molecule, then H-1*ax* (δ= 2.38 ppm) and H-1*eq* (ddd at δ= 2.74 ppm, J= 16.2, 3, and 2 Hz) could be assigned. In addition, the latter signals led to ^1H-^{13}C long range correlations to the respective carbon signals of both CO groups. Examination of the coupling constants in the 2D-phase sensitive DFQCOSY showed that H-7a is equatorial with respect to the 6-membered ring, as demonstrated by a small (10 Hz) half-height width for this signal and a small (4 Hz) coupling constant for H-1*ax*. The *cis*-junction of the rings was confirmed by 2D phase sensitive NOESY experiments (600 ms mixing time) indicating an effect between H-7a and the methyl group (Figure 1).



2 **5** **6**

Figure 1: Major Nuclear Overhauser Effects Observed by 2D-NOESY or Saturation of the Axial Methyl Group or H-1 or H-7a Signals. Minimal energy conformations have been determined using the Alchemy III® (Tripos Ass.Inc.) program on a Mac II computer.

In a preparative 2 days-incubation of *S*-**1**, 12% of the substrate was recovered unchanged, while an unseparated mixture (51%) of the two major hydroxylated products **A** [= (4a*S*,7*S*)-*trans*-**2**] and **B** [= (4a*S*,7*R*)-*cis*-**3**] was isolated (2:8 ratio) from the incubation filtrate (Scheme 2). From a 10 days-incubation in the culture medium (total conversion), the same hydroxylated products were isolated in equal amounts (19% total yield), together with a new product **6** (17%). A small amount (about 1%) of another new product **7** was also isolated.

Similar results were obtained by using the washed mycelium resuspended in a 50 mM potassium phosphate buffer pH 7.0. Kinetic studies by HPLC (Figure 2) clearly indicated that the reduced product **6** was derived from the initial major product **B**, while the minor (4a*S*,7*S*)-*trans*-**2** was not affected by this transformation.

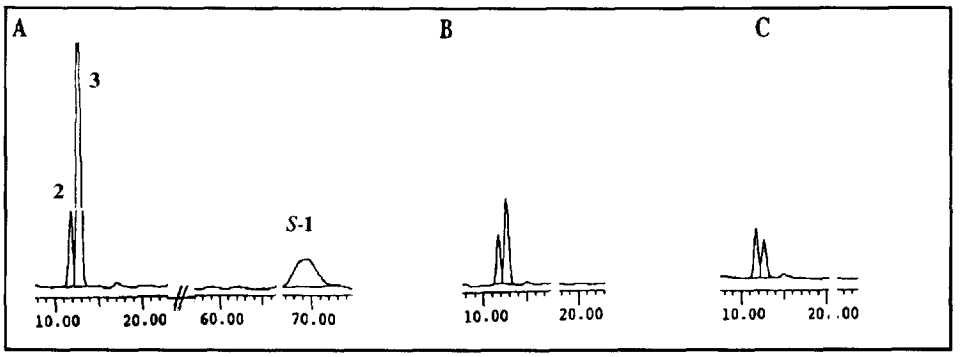
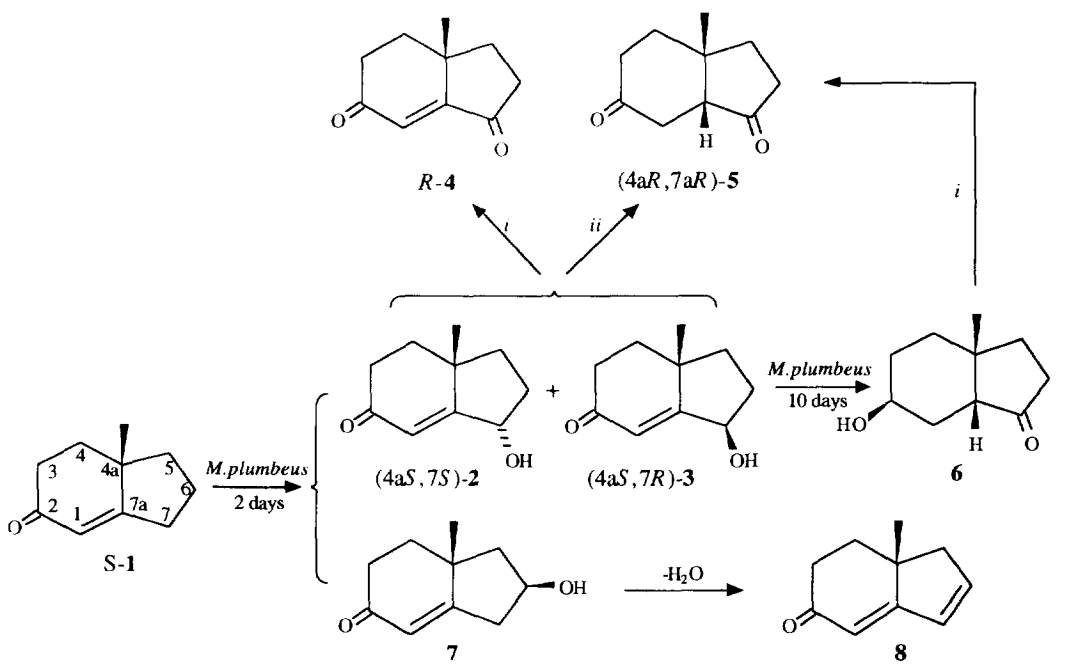


Figure 2: HPLC profiles at 245 nm (see experimental) of 2 days- (*left*), 7 days- (*center*), and 10 days-incubations (*right*) of hydrindenone *S*-**1** with *M. plumbeus*.

A (4a*S*,7*R*)-*cis*-7-hydroxy structure **3** was assigned to product **B**, which could not be completely separated from **2** by repeated chromatography. However, the structure of **3** could be analyzed by NMR techniques on the **2+3** mixture, as NMR features of **2** have been previously determined on its pure (4a*R*,7*R*)-enantiomer, produced from *R*-**1**. Beside a characteristic C*H*OH signal at 4.69 ppm, a significantly downfield shift was found for the 4a-methyl group signal ($\Delta\delta = +0.15$ ppm, with respect to **1**), indicative of a *cis*-configuration. Jones oxidation of the **2+3** mixture afforded a single pure diketone **4**, enantiomer of that obtained from the hydroxylation product of *R*-**1**. Acidic rearrangement of the **2+3** mixture led to a single diketo saturated product **5**, enantiomer of that obtained from the hydroxylation product of *R*-**1**. These results clearly indicate that *M.plumbeus* produces from the *S*-enantiomer of **1** a diastereomeric mixture of *cis*- and *trans*-7-hydroxylated products, with the *cis* isomer predominating, while it produced exclusively the *trans*-7-hydroxylated product from the *R*-enantiomer.



Scheme 2: *i*) Air oxidation, or CrO_3, H_2SO_4/acetone; *ii*) HBr /Et_2O

The elucidation of the structure of compound **6**, derived from **3**, confirmed the above assignments. No vinylic proton was detected by ^1H NMR in the 5-6 ppm region, indicating a reduction of the 1,7a-conjugated double bond of **3**, confirmed by a loss of UV absorption in the 250 nm region. A *CH*OH signal at 3.36 ppm, appearing as a triplet of triplets with a large *ax-ax* coupling constant (about 11 Hz) and a small *ax-eq* coupling constant (about 4 Hz), suggested an equatorial OH group with two vicinal CH_2 groups. Long range ^1H-^{13}C correlation (PFG-HMBC) from CH_3 signal led to the assignment of CH_2-4 and-5. COSY experiment showed that the protons belonging to these two CH_2 groups and H-7a (assigned unambiguously from ^1H-^{13}C correlation) are not coupled to the *CH*OH signal. Thus, the hydroxyl and carbonyl groups could be only located on C-2 and C-6 respectively. Moreover, H-7a signal exhibited a small (11 Hz) half-height width, in agreement with an equatorial position of H-7a, with respect to the 6-membered ring, and demonstrating a *cis*-junction of the rings. This was confirmed by nOe experiments (2D phase sensitive NOESY, 600ms mixing time) with an effect between the methyl group and H-7a (Figure 1). The fact that no nOe's of these signals and *CH*OH signal could be observed confirmed the equatorial position of the 2-OH group. The Jones oxidation of **6** afforded quantitatively the saturated diketone **5**, previously prepared by acidic rearragement of **2** (or **2+3**).

Product **7**, a very minor product of the incubation of *R*-**1** with *M. plumbeus*, was easily identified as a 6-hydroxylated derivative by ^1H (*CH*OH signal as a multiplet at 4.52 ppm, and 4a-methyl group at 1.42 ppm indicative of a strong *cis* -1,3-diaxial interaction), ^{13}C NMR (CHOH at 70.70 ppm) and ^1H,^{13}C correlation spectra. This compound slowly dehydrated to give the corresponding conjugated dienic ketone **8**, identified by mass spectrometry and ^1H and ^{13}C NMR.

As shown in Table 1, other strains metabolize both enantiomers of **1** . The alternative use of *B. bassiana* in short incubations with *R*-**1** may allow the preparation of both diastereomeric 7-hydroxy derivatives (4a*R*,7*R*)-**2** and (4a*R*,7*S*)-**3** in moderate yields. With regard to *S*-**1**, a biotransformation pattern very similar to that observed with *M. plumbeus* was found in the incubations with *B. bassiana* and *C. lunata* ; the faster and selective reduction of (4a*S*,7*R*)-**3** to (2*S*,4a*R*,7a*R*)-**6** in prolonged incubations of *S*-**1** with *C. lunata* may allow to obtain pure residual (4a ,7*S*)-**2** in moderate amounts.

CONCLUSION

It is remarkable that in the hydrindenone series, oppositely to the hydronaphthalenone one [16], a *trans* allylic hydroxylation of the *R-enantiomer* occurs exclusively, while a major *cis* hydroxylated compound is formed from the *S-enantiomer*, using the same microorganism (*M. plumbeus).*

These results show again that, despite the formation of some by-products, due to other enzymic activities of the whole microorganism used, valuable new regio- and stereoselectively functionalized derivatives of easily prepared bicyclic enones can be obtained in appreciable yields by microbial hydroxylation. Work is in progress to extend these experiments to a wide range of other substituted hydrindenones.

EXPERIMENTAL

General . Melting points were determined in capillary tubes and are uncorrected. ^1H-NMR spectra were generally obtained in $CDCl_3$ at 250 MHz on a WM 250 Bruker spectrometer and occasionnally on an AMX 500 Bruker spectrometer at 500 MHz. Chemical shifts are expressed in ppm from tetramethylsilane (using as a reference residual $CHCl_3$). Multiplicities are reported as br. (broad), s (singlet), d (doublet), t (triplet) q (quadruplet) and m (multiplet). ^{13}C-NMR spectra were recorded at 62.9 MHz and multiplicities were assigned by polarization transfer using a DEPT 135 sequence. 2D-^1H,^1H COSY NMR spectra were acquired at 250 or 500 MHz. Combined pulse field gradient double quantum filter COSY (PFG-DQFCOSY) [20], heteronuclear multiquantum correlation (PFG-HMQC), and heteronuclear multiple bond correlation (PFG-HMBC) [21] spectra were performed at 500 MHz. TLC on Merck $60F_{254}$ precoated plates, HPLC and GC were routinely used to monitor the bioconversion experiments. A DBWax capillary column (0.2 mm x 30 m) was used on a Shimadzu G6 GC

instrument equipped with a flame ionization detector and a Shimadzu CR-6A integrating-recorder. HPLC was performed at 1mL min^{-1} on a Lichrospher 100 RP-18 column (Merck, 4.5 mm x 25 cm.) and detection at 245 nm with a Shimadzu SPD-6A. Enantiomeric purity of the hydrindenone substrates was determined or verified by HPLC on a Chiralpak AD column (Daicel, 4.5 mm x 25 cm); solvent heptane-iPrOH, 99:1, 0.5 mL min^{-1}. Flash chromatography on silica gel (Merck 60, 230-400 mesh) was used for products separation with cyclohexane-EtOAc (6:4) as solvent. Optical rotations were measured in a 1 dm-cell on a Perkin Elmer 241 spectropolarimeter. GC-MS analyses were carried out by electronic impact on a 5890-II/5972 Hewlett-Packard instrument equipped with a HP-1 (30 m) capillary column. Mass spectra (EI mode) and high resolution mass measurements (HRMS) were supplied by the University Pierre and Marie Curie (Paris), Spectrochemistry Centre. Commercial reagents and solvents were used without further purification.

Starting materials. (*S*)-(+) and (*R*)-(−)-4a-methyl-4,4a,5,6-tetrahydro-2(3*H*)-indenone (**1**) enantiomers were synthesized as previously described [1,22] and crystallized at low temperature in heptane [9]; $[\alpha]_D^{21}$= +111 (c 1.04, EtOH), 94% ee, and $[\alpha]_D^{21}$= −118 (c 1.1, EtOH), ee 98%, respectively . ^{1}H-NMR, δ ppm, J Hz: 5.75 (t, J=2.2, H-1), 2.68 (ddm, J=18 and 9, H-7β), 2.54 (ddd, J=18, 14.5 and 5, H-3ax), 2.4 (m, H-7α), 2.31 (dddd, J=18, 5.2, 2.2 and 1, H-3eq), 1.98 (ddd, J=13.2, 5.4 and 2.2, H-4eq), 1.7-1.9 (m, H-4ax, H-5β, H-6α and H-6β), 1.45 (dt, J=8 and 11.5, H-5α), 1.14 (s, CH_3-4a). ^{13}C-NMR, δ ppm: 199.50 (C-2), 178.45 (C-7a), 121.26 (C-1), 42.65 (C-4a), 40.82 (C-5), 36.06 (C-4), 33.78 (C-3), 30.71 (C-7), 22.38 (CH_3-4a), 21.13 (C-6).

Microorganisms, culture and incubation conditions. Screening of microorganisms from international collections was effected using a standard liquid medium [16] (100 mL) in orbitally shaken (200 r.p.m.) conical flasks, inoculated with spores freshly obtained from a solid medium, and incubated for 60-72 hours at 27°C in order to obtain maximal growth; oily substrates (50 mg) were directly added to the cultures, sometimes after dilution into a small volume of ethanol, and incubation was continued in the same conditions. Progress of the biotransformation was followed by HPLC of a sample of incubation filtrate, and by TLC and GC of a sample of incubation filtrate extracted with ethyl acetate.

Preparative incubations were effected either through a simple scaling up of the screening procedure, in 2 L conical flasks with built-in deflectors, containing 0.75 L of culture medium or using a pelleted mycelium previously grown in a Biolafitte fermentor (7.5 L), filtered and washed with tap water then resuspended in a 0.1M potassium phosphate buffer, pH 7.0 (about 20 g dry weight mycelium L^{-1}) to which the substrate (0.5-1 g L^{-1}) was added.

Isolation and purification of biotransformation products. The incubation medium was filtered on filter paper with celite and the filtrate, saturated with sodium chloride (and eventually filtered again) was repeatedly extracted with ethyl ether-ethyl acetate (1:1). In most cases, owing to the large amount of material extracted, compared with the minute amount of useful product detected, the mycelium extract was discarded; in some few cases, it was extracted apart and the extract pooled with the filtrate extract for further purification.

Biotransformation of (R)-4a-methyl-4,4a,5,6-tetrahydro-2(3H)-indenone (*R*-**1**). After a 10-days incubation (300 mg, 0.5 g L^{-1}) with *M.plumbeus* CBS 110-16, filtration, extraction with EtOAc, and flash chromatography, 30 mg (10%) of the substrate was recovered followed by 155 mg (47%) of (4a*R*,7*R*)-**2** as a colorless oil, $[\alpha]_D^{21}$= −102 (c 1.16, $CHCl_3$). HRMS for $C_{10}H_{14}O_2$, calc.166.0994, found 166.0933; MS (EI): 166(21) $[M]^+$, 138(20) $[M\text{-}CO]^+$, 123(12) $[138\text{-}CH_3]$, 110(100) $[M\text{-}2CO]^+$, 93(75); ^{1}H-NMR, δ ppm, J Hz: 5.92 (d, J = 1.5, H-1), 4.80 (ddd, J = 8.4, 6.4 and 1.5, H-7α), 2.52 (ddd, J=18, 13.5 and 5.5, H-3ax), 2.35 (ddd, J=18, 13.5 and 6, H-3eq), 2.3 (m, H-6α), 1.98 (m, H-4eq), 1.89 (dt, J=5 and 13.5, H-4ax), 1.6-1.8 (m, H-5α, H-5β and H-6β), 1.19 (s, CH_3-4a). ^{13}C-NMR, δ ppm: 200.30 (C-2), 179.28 (C-7a), 119.32 (C-1), 72.37 (C-7), 39.91 (C-4a), 37.20, 31.53 (C-5 and C-6), 36.99 (C-4), 33.93 (C-3), 24.59 (CH_3-4a).

*Biotransformation of (S)-4a-methyl-4,4a,5,6-tetrahydro-2(3H)-indenone (S-**1**).*

a) After a 2-days incubation in the culture medium (380 mg, 0.5 g L^{-1}) with *M.plumbeus* CBS 110-16, filtration, extraction with EtOAc, and medium pressure chromatography, 14 mg (4%) of the substrate was recovered, followed by a mixture (216 mg, 51%) of (4a*S*,7*S*)-**2** and (4a*S*,7*R*)-**3** (2:8). A small amount of **6** (14 mg, 3%) was also obtained.

b) After a 10-days incubation in the culture medium (380 mg, 0.5 g L^{-1}) with *M.plumbeus* CBS 110-16, filtration, extraction with EtOAc, flash chromatography and purification of fractions by preparative HPLC (solvent: MeOH-H_2O, 45:55), 79 mg (19%) of **6**, 5 mg (1%) of **7**, and a mixture (72 mg, 17%) of (4a*S*,7*S*)-**2** and (4a*S*,7*R*)-**3** (1:1) were obtained. ^{1}H- and ^{13}C-NMR data for (4a*S*,7*R*)-**3** were deduced from analyses of the mixture of **2+3** and comparison with NMR data of (4a*R*,7*R*)-**2** (see above). ^{1}H-NMR, δ ppm: 5.93 (s,H-1), 4.69 (CH-7α), 2.20-2.48 and 1.80-2.05 (H-6), 1.31 (s, CH_3-4a). ^{13}C-NMR, δ ppm: 200.41 (C-2), 176.39 (C-7a), 122.93 (C-1), 74.15 (C-7), 40.06 (C-4a), 37.83, 33.85 (C-5 and C-6), 37.00 (C-4), 33.85 (C-3), 23.44 (CH_3-4a).

6, colorless oil, $[\alpha]_D^{21}$= –127.5 (c 1.6, $CHCl_3$). HRMS for $C_{10}H_{16}O_2$, calc.168.1150, found 168.1153; MS (EI): 168(3) $[M]^+$, 150(35) $[M-H_2O]^+$, 97(100); ^{1}H-NMR, δ ppm, J Hz: 3.36 (1H, tt, J= 4 and 10.5, H-2*ax*), 2.25-2.30 (3H, m, H-1*eq* and H-6), 2.01 (1H, m, H-7a), 1.78 (1H, m, H-5α), 1.71 (1H, m, H-3*eq*), 1.66 (1H, m, H-5β), 1.45 (1H, m, H-1*ax*), 1.38 (1H, m, H-3ax), 1.26 (1H, m, H-4*ax*), 1.22 (3H, s, CH_3-4a). ^{13}C-NMR, δ ppm: 219.29 (C-7), 67.51 (C-2), 56.13 (C-7a), 37.71 (C-4a), 34.87 (C-6), 34.09 (C-5), 31.87 (C-4), 30.53 (C-3), 29.92 (C-1), 25.44 (CH_3-4a).

7, colorless oil. MS (EI): 166(25) $[M]^+$, 148(17) $[M-H_2O]^+$, 138(100) $[M-CO]^+$, 124(70), 120(39), 107(35), 95(44). ^{1}H-NMR, δ ppm, J Hz: 5.74 (1H, br.s, H-1), 4.52 (1H, m, H-6α), 2.91 (1H, ddd, J = 18.7, 6.8 and 1, H-7β), 2.75 (1H, br.d, H-7α), 2.54 (1H, ddd, J = 17.9, 14.4 and 5.6, H-3*ax*), 2.34 (1H, ddd, J= 17.9, 4.8 and 1.6, H-3*eq*), 2.05 (1H, m, H-4*eq*), 1.86 (2H, m, H-5), 1.80 (1H, m, H-4*ax*), 1.41 (3H, s, CH_3-4a). ^{13}C-NMR, δ ppm: 199.06 (C-2), 175.84 (C-7a), 121.66 (C-1), 50.20 (C-5), 70.70 (C-6), 42.06 (C-7), 41.78 (C-4a), 36.64 (C-4), 33.83 (C-3), 24.69 (CH_3-4a).

On keeping in $CHCl_3$ solution at room temperature, **7** was slowly dehydrated to **8**. MS (EI): 148(45) $[M]^+$, 120(100), 105(10), 91(80). ^{1}H-NMR, δ ppm, J Hz: 6.54 (1H, m, H-6), 6.33 (1H, m, H-7), 5.80 (1H, s, H-1), 2.55 (1H, m, H-3*eq*), 2.42 (3H, m, H-3*ax* and H-5), 2.05 (1H, m, H-4*eq*), 1.79 (1H, m, H-4*ax*), 1.22 (3H, s, CH_3-4a). ^{13}C-NMR, δ ppm: 203.88 (C-2), 175.84 (C-7a), 146,42 (C-6), 131.08 (C-7), 117.71 (C-1), 48.10 (C-5), 29.68 (C-4a), 36.64 (C-4), 36.31 (C-3), 26.05 (CH_3-4a).

c) After a 10-days incubation in buffer, (360 mg, 0.5 g L^{-1}) with a washed mycelium of *M.plumbeus* CBS 110-16, filtration, extraction with EtOAc, and flash chromatography, 110 mg of unchanged substrate (31%), 68 mg (19%) of (4a*S*,7*S*)-**2** and (4a*S*,7*R*)-**3**, and 114 mg of **6** (31%) were obtained.

Oxidation of **2** (or **2+3**) to *4a-methyl-4,4a,5,6-tetrahydro-2(3H),7-indendione* (**4**). (4a*R*,7*R*)-**2** (27 mg) in 1 mL of acetone was treated at 0°C with 60 µl of a solution of chromic anhydride (1 g) in water-H_2SO_4 (5 mL, 4:1). After stirring for 30 min, water was added, acetone was evaporated *in vacuo* and the mixture was extracted with ethyl ether. Preparative TLC (cyclohexane-EtOac, 7:3) and recrystallisation in hexane afforded 14 mg of (*S*)-**4** as pale yellow crystals, m.p.= 73°C, $[\alpha]_D^{21}$= –330 (c 0.7, $CHCl_3$). HRMS for $C_{10}H_{12}O_2$, calc.164.0837, found 164.0814; MS (EI): 164(40) $[M]^+$, 136(100) $[M-CO]^+$; ^{1}H-NMR, δ ppm, J Hz: 6.26 (s, $w_{1/2}$=2, H-1), 2.4-2.7 (m, H-3*ax*, H-3*eq*, H-6α and H-6β), 2.17 (ddd, J=13.5, 5.1 and 2.3, H-4eq), 2.12 (ddd, J=12.5, 4.2 and 1.8, H-5β), 2.01 (dt, J=5.4 and 13.5, H-4*ax*), 1.75 (dt, J=8.8 and 12.5, H-5α), 1.31 (s, CH_3-4a); ^{13}C-NMR, δ ppm: 207.11, 199.99 (C-2 and C-7), 160.65 (C-7a), 122.65 (C-1), 39.73 (C-4a), 37.12 (C-5), 36.23 (C-4), 35.33 (C-6), 34.11 (C-3), 22.90 (CH_3-4a).

A similar reaction on a mixture of (4a*S*,7*S*)-**2** and (4a*S*,7*R*)-**3** (30 mg, 2:8) afforded 14 mg of the enantiomeric diketone (*R*)-**4**, $[\alpha]_D^{21}$= +332 (c 0.68, $CHCl_3$).

Acidic isomerization [18] *of* **2** *to cis-4a-methyl-1,7a,4,4a-tetrahydro-2(3H),7-indandione* (**5**). (4a*R*,7*R*)-**2** (20 mg) in 1 mL of ethyl ether was treated at room temperature with 3 drops of concentrated (47%) HBr and stirred for 2 hours. After neutralization with triethylamine, dilution with water, and repeated extraction with ether, preparative TLC (cyclohexane-EtOac, 7:3) afforded (4a*S*,7a*S*)-**5** (9 mg), m.p.= 99.5°C, $[\alpha]_D^{21}$= +152 (c 0.47, $CHCl_3$). HRMS for $C_{10}H_{14}O_2$, calc.166.0993, found 166.0990. MS (EI): 166(98) $[M]^+$, 151(3) $[M\text{-}CH_3]^+$, 148(1) $[M\text{-}H_2O]^+$, 70(100); ^{1}H-NMR, δ ppm, J Hz: 2.74 (1H, ddd, J=16.2, 3, and 2, H-1*eq*), 2.25-2.40 (6H, m, H-1*ax*, H-3, H-6, H-7a), 1.85-1.92 (2H, m, H-5), 1.70 (2H, m, H-4), 1.31 (3H, s, CH_3-4a); ^{13}C-NMR, δ ppm: 216.77 and 208.66 (C-7 and C-2), 56.31 (C-7a), 37.69 (C-4a), 36.88 (C-3), 35.65 (C-1), 34.61 (C-6), 33.84 (C-5), 33.10 (C-4), 25.22 (CH_3-4a).
A similar reaction on a mixture of (4a*S*,7*S*)-**2** and (4a*S*,7*R*)-**3** (21 mg, 2:8) afforded 11 mg of the enantiomeric diketone (4a*R*,7a*R*)-**5**, $[\alpha]_D^{21}$= −153 (c 0.56, $CHCl_3$).

Oxidation of **6** *to (4aR,7aR)-4a-methyl-1,7a,4,4a-tetrahydro-2(3H),7-indandione* (**5**). (2*S*, 4a*R*,7a*R*)-**6** (11 mg) was oxidized in acetone as described above to give, after TLC purification, 6 mg of (4a*R*,7a*R*)-**5**.

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