Microbial Transformation of Isolongifolen-4-one

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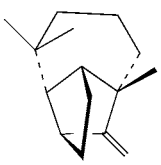
The microbial transformation of (+)-isolongifolen-4-one (**4**) by a number of fungi by means of a standard two-stage fermentation technique afforded (7*R*)-12-hydroxyisolongifolen-4-one (**5**), (7*S*)-13-hydroxyisolongifolen-4-one (**6**), (11*R*)-11-hydroxyisolongifolen-4-one (**7**), (10*R*)-10-hydroxyisolongifolen-4-one (**8**), and (9*R*)-9-hydroxyisolongifolen-4-one (**9**) (*Scheme*). All five metabolites were found to be new, and metabolites **6** and **9** showed potent tyrosinase inhibitory activity (*Table 1*). The metabolites and their derivatives were characterized on the basis of spectroscopic and single-crystal X-ray-diffraction techniques.

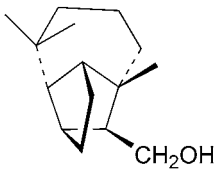
Introduction. – Tyrosinase is a multifunctional copper-containing enzyme widely distributed in plants and animals. It is known to be a key enzyme for melanin biosynthesis in plants and animals. The developments of tyrosinase inhibitors is, therefore, clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation, and these inhibitors are also often added in cosmetics for skin whitening and depigmentation after sunburn [1]. Recently, a number of natural inhibitors of tyrosinase have been introduced in cosmetics [2].

In continuation of our studies on microbial transformation of pharmacologically important compounds [3–10], we have recently screened longifolene (**1**) and its derivatives isolongifolene (**2**), isolongifolol (**3**), and isolongifolen-4-one (**4**) against tyrosinase. Compound **4** was found to be active against tyrosinase ($IC_{50} = 51$ μm). Therefore, we carried out derivatization of compound **4** with the aim to find derivatives with increased potency and to understand the structure-activity relationships. Longifolene (=(1*S*,3a*R*,4*S*,8a*S*)-decahydro-4,8,8-trimethyl-9-methylene-1,4-methanoazulene; **1**) is a bridged tricyclic sesquiterpene, found in terpentine oil, which is produced commercially from the oleoresin of the Himalayan pine, *Pinus longifolia* ROXB. [11].

We exposed compound **4** to four fungal cultures, which yielded the three major metabolites **5**, **6**, and **9** and the two minor metabolites **7** and **8** (*Scheme*). The major metabolites were obtained in reasonable yields (>20%) and were subjected to chemical derivatization (**10**–**18**) and an enzyme inhibitory assay. Metabolites **6** and **9** were found to be potent inhibitors of tyrosinase (*Table 1*), while the benzyl ether derivative **17** of metabolite **9** was an even more potent inhibitor of the enzyme (*Table 1*).



1



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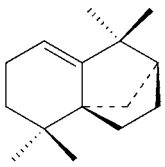

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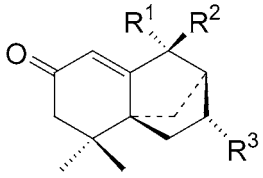
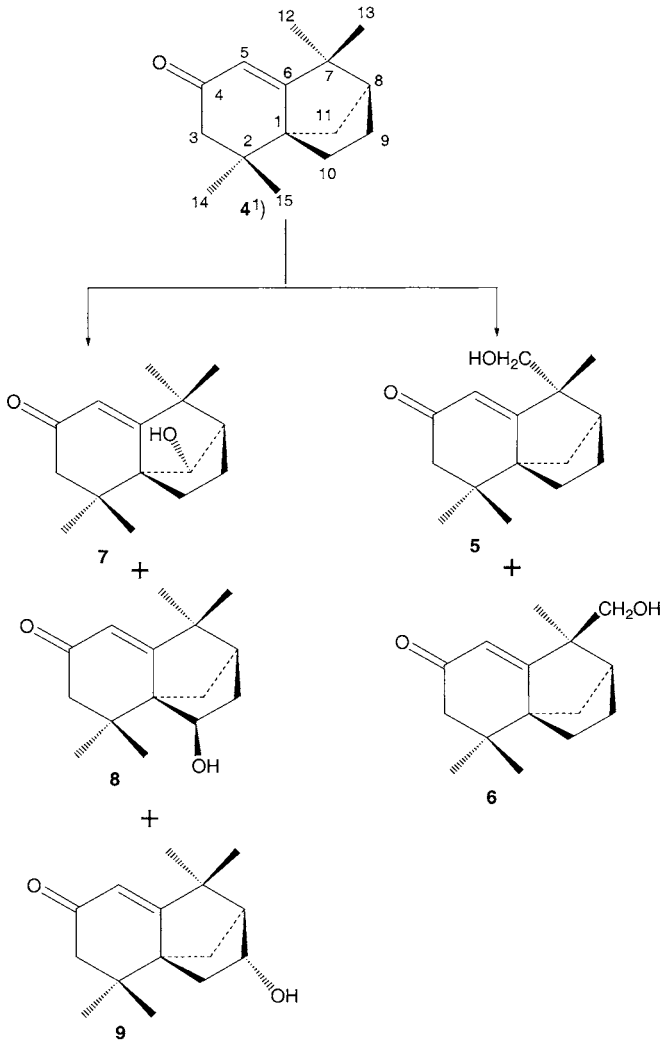


10 R^1 = $AcOCH_2$, R^2 = Me, R^3= H
11 R^1 = $MeOCH_2$, R^2 = Me, R^3 = H
12 R^1 = Me, R^2 = $AcOCH_2$, R^3 = H
13 R^1 = Me, R^2 = $MeOCH_2$, R^3 = H
14 R^1 = R^2 = Me, R^3 = AcO
15 R^1 = R^2 = Me, R^3 = MeO
16 R^1 = R^2 = Me, R^3 = EtO
17 R^1 = R^2 = Me, R^3 = BnO
18 R^1 = R^2 = Me, R^3 = (C=O)

Results and Discussion. – Small-scale experiments showed that all four fungal cultures were capable of converting compound **4** into polar metabolites. Preparative-scale fermentations were thus carried out to produce sufficient quantities of metabolites for biological studies and chemical derivatization. Metabolites **5**–**7** were produced by *Aspergillus niger* (ATCC 10549), while metabolites **8** and **9** were formed by *Fusarium lini* (NRRL 68751). Similarly, *Cephalosporium aphidicola* (IMI 68689) afforded metabolite **9**, while *Rhizopus stolonifer* (ATCC 10404) afforded metabolites **5** and **6**. The structures of metabolites **5**–**9** were elucidated by spectroscopic studies and comparison of the spectra with those of substrate **4**.

Metabolite **5**, obtained as colorless crystals, exhibited the M^+ signal at m/z 234.1617 ($C_{15}H_{22}O_2^+$) in the HR-EI-MS, 16 amu higher than M^+ of **4**. Another significant ion was at m/z 203.1424 ($C_{14}H_{20}O^+$) due to $[M - CH_2OH]^+$. The IR spectrum showed an additional absorption at 3392 cm^{-1} (OH) in comparison to the IR spectrum of **4**. Further spectral data (see *Tables 2* and *3* for ^{1}H- and ^{13}C-NMR data) established the

Scheme. *Oxidative Metabolism of Isolongifolen-4-one*[1]) (**4**)



structure of the metabolite as (7*R*)-12-hydroxyisolongifolen-4-one (**5**)[1]). The configuration at C(7) was determined to be (*R*) by a single-crystal X-ray-analysis (*Fig. 1*).

The ^{1}H-NMR spectrum of **5** exhibited additional downfield signals at δ 3.47 (*d*, $J = 11.0$ Hz, 1 H) and 3.33 (*d*, $J = 11.0$ Hz, 1 H) arising from diastereotopic protons of an OH-bearing CH_2 group and the disappearance of the Me(12) signal of the geminal $Me_2C(7)$ moiety of **4**, indicating oxygenation at C(12) of **5**. The appearance of the additional O-bearing CH_2 at δ 67.6 in the DEPT experiment further supported structure **5**. Moreover, H_a–C(12) (δ 3.47) and H_b–C(12) (δ 3.33) showed cross-coupling with each other in the COSY-45° spectrum

[1]) Arbitrary numbering; for systematic names, see *Exper. Part.*

Table 1. *Tyrosinase Inhibitory Activity of Compounds* **1**–**6** *and* **9**–**18**[a])

Compound	IC_{50} (in μM ± s.e.m.)
1	n.a.[b])
2	n.a.[b])
3	520.56 ± 0.588
4	51.91 ± 0.0245
5	101.01 ± 0.1978
6	9.64 ± 0.0008
9	6.68 ± 0.0096
10	14.05 ± 5.675
11	22.03 ± 0.0011
12	7.45 ± 0.0443
13	n.a.[b])
14	68.79 ± 0.8704
15	5.79 ± 0.0017
16	9.77 ± 0.0046
17	2.70 ± 0.0026
18	n.a.[b])
Kojic acid[c])	16.92 ± 0.5268
L-Mimosine[c])	3.68 ± 0.0223

[a]) Metabolites **7** and **8** could not be screened because of their insufficient amount; s.e.m. = standard error of the mean. [b]) n.a. = Not active. [c]) Standard inhibitors of the enzyme tyrosinase.

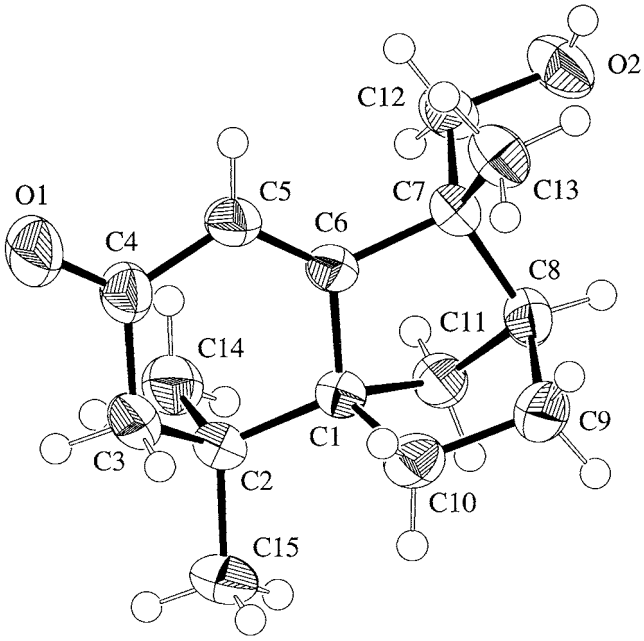


Fig. 1. *ORTEP Drawing of the X-ray crystal structure of* **5**[l])

Table 2. *^{1}H-NMR Data for Compounds* **4**–**9** (500 MHz, $CDCl_3$)[1])

	4	**5**	**6**	**7**	**8**	**9**
CH_2(3)	2.35, 2.05 (2*d*, *J* = 16.2)	2.35, 2.02 (2*d*, *J* = 16.2)	2.35, 2.06 (2*d*, *J* = 16.2)	2.59, 2.14 (2*d*, *J* = 16.7)	2.82, 1.98 (2*d*, *J* = 16.5)	2.32, 2.05 (2*d*, *J* = 16.2)
H−C(5)	5.67 (*s*)	5.67 (br. *s*)	5.67 (br. *s*)	5.67 (br. *s*)	5.67 (br. *s*)	5.67 (br. *s*)
H−C(8)	1.94 (*t*, *J* = 3.4)	2.19 (*t*, *J* = 2.0)	2.19 (*t*, *J* = 2.0)	1.97 (*d*, *J* = 4.4)	2.1 (br. *s*)	1.95 (br. *s*)
CH_2(9) or H−C(9)	1.90 (*dd*, *J* = 12.1, 3.9), 1.74 (*m*)	1.73, 1.64 (2*m*)	1.76, 1.65 (2*m*)	1.75, 1.63 (2*m*)	1.85, 1.76 (2*m*)	4.32 (*d*, *J* = 6.5)
CH_2(10) or H−C(10)	1.57, 1.37 (2*m*)	1.59, 1.38 (2*m*)	1.58, 1.42 (2*m*)	1.92, 1.79 (2*m*)	4.46 (*dd*, *J* = 10.2, 2.9)	1.77 (*dd*, *J* = 6.5, 2.5), 1.74 (*dd*, *J* = 1.0, 2.4)
CH_2(11) or H−C(11)	1.66, 1.47 (2*m*)	1.95 (*td*, *J* = 8.2)	1.95 (*td*, *J* = 8.4)	3.95 (*d*, *J* = 4.6)	1.67, 1.53 (2*m*)	1.88 (*dd*, *J* = 1.52, 10.4), 1.65 (*m*)
Me(12) or CH_2(12)	1.11 (*s*)	3.47, 3.33 (2*d*, *J* = 11.0)	0.99[a]) (*s*)	1.06[a]) (*s*)	1.10 (*s*)	1.06[a]) (*s*)
Me(13) or CH_2(13)	1.05[a]) (*s*)	0.9 (*s*)	3.61, 3.53 (2*d*, *J* = 10.9)	0.98 (*s*)	0.95 (*s*)	0.98 (*s*)
Me(14)	1.01[a]) (*s*)	1.17 (*s*)	1.17 (*s*)	1.22 (*s*)	1.19[a]) (*s*)	1.11 (*s*)
Me(15)	0.96 (*s*)	1.0 (*s*)	1.04[a]) (*s*)	1.07[a]) (*s*)	1.14[a]) (*s*)	1.09[a]) (*s*)

[a]) These values can be interchanged.

Table 3. *^{13}C-NMR Data for Compounds* **4**–**9** (125 MHz, $CDCl_3$). Multiplicities were determined by DEPT experiments.

	4	**5**	**6**	**7**	**8**	**9**
C(1)	60.2 (*s*)	58.6 (*s*)	58.6 (*s*)	60.1 (*s*)	59.8 (*s*)	57.9 (*s*)
C(2)	35.5 (*s*)	34.6 (*s*)	34.3 (*s*)	34.2 (*s*)	33.4 (*s*)	32.1 (*s*)
C(3)	50.7 (*t*)	49.9 (*t*)	50.0 (*t*)	50.1 (*t*)	53.3 (*t*)	49.8 (*t*)
C(4)	202.9 (*s*)	199.9 (*s*)	199.2 (*s*)	199.6 (*s*)	200.1 (*s*)	200.0 (*s*)
C(5)	117.3 (*d*)	118.6 (*d*)	118.0 (*d*)	118.2 (*d*)	120.7 (*d*)	117.6 (*d*)
C(6)	187.6 (*s*)	179.2 (*s*)	179.6 (*s*)	179.2 (*s*)	179.6 (*s*)	181.5 (*s*)
C(7)	45.3 (*s*)	49.7 (*s*)	49.3 (*s*)	42.0 (*s*)	41.3 (*s*)	42.1 (*s*)
C(8)	47.9 (*d*)	42.1 (*d*)	43.2 (*d*)	55.2 (*d*)	43.0 (*d*)	54.4 (*d*)
C(9)	25.2 (*t*)	24.3 (*t*)	24.3 (*t*)	25.7 (*t*)	42.1 (*t*)	70.3 (*d*)
C(10)	37.6 (*t*)	36.4 (*t*)	36.5 (*t*)	35.9 (*t*)	72.1 (*d*)	40.2 (*t*)
C(11)	28.9 (*t*)	28.7 (*t*)	28.1 (*t*)	75.2 (*d*)	30.1 (*t*)	32.8 (*t*)
C(12)	27.2 (*q*)	67.6 (*t*)	22.0 (*q*)	25.7[a] (*q*)	25.1[a]) (*q*)	25.4[a]) (*q*)
C(13)	25.6 (*q*)	19.9 (*q*)	67.4 (*t*)	25.1[a]) (*q*)	23.3 (*q*)	24.2 (*q*)
C(14)	25.9 (*q*)	25.8[a]) (*q*)	25.9[a]) (*q*)	27.1 (*q*)	26.7 (*q*)	27.2 (*q*)
C(15)	24.8 (*q*)	25.5[a]) (*q*)	25.3[a]) (*q*)	25.9 (*q*)	25.5[a]) (*q*)	25.9[a]) (*q*)

[a]) These values can be interchanged.

and heteronuclear couplings with C(13) (δ 19.9) and C(12) (δ 67.6); H_b–C(12) also showed a ^2J(C,H) coupling with C(7) (δ 49.7) in the HMBC spectrum.

The HR-EI-MS of metabolite **6** exhibited M^+ at m/z 234.1613, corresponding to the molecular formula $C_{15}H_{22}O_2$ (calc. 234.1620). The loss of CH_2OH from the molecular ion gave rise to m/z 203.1432 ($C_{14}H_{20}O^+$). The IR spectrum again showed OH absorptions (3419 cm^{-1}). Metabolite **6** was found to be the 7-epimer of **5**.

The absence of one Me signal of the geminal $Me_2C(7)$ moiety of **4** in the ^{1}H-NMR spectrum of compound **6** (*Table 2*), and the presence of an *AB* signal for CH_2OH at δ 3.61 ($J = 10.95$, H_A) and 3.53 ($J = 10.95$, H_B), as well as the appearance of a new CH_2 signal at δ 67.4 in the ^{13}C-NMR spectrum (*Table 3*) suggested that **6** was an epimer of **5**. The configuration at C(7) of **6** as (*S*) was inferred from the NOE experiment: when the signals of CH_2OH (δ 3.61 and 3.53) were irradiated, an enhancement of the signal of the β-oriented Me(15) (4.3%) was observed, indicating the β-position of the CH_2OH group at C(7). The single-crystal X-ray-diffraction experiment with **5** was also useful to differentiate between the two epimers.

The HR-EI-MS of metabolite **7** exhibited M^+ at m/z 234.1618 ($C_{15}H_{22}O_2^+$), in addition to m/z 203.8765 ($C_{15}H_{22}O_2^+$) for $[M - H_2O]^+$. The IR spectrum displayed OH absorptions at 3442 cm^{-1}. The structure of **7** was deduced as (11*R*)-11-hydroxyisolongifolen-4-one.

A characteristic *d* in the ^{1}H-NMR spectrum of **7** (*Table 2*) at δ 3.95 ($J = 4.6$ Hz) was assigned to H–C(11) geminal to the newly introduced OH group. The ^{13}C-NMR spectrum (*Table 3*) displayed signals for four Me, three CH_2, three CH, and five quaternary C-atoms. A CH signal resonating at δ 75.2 was very informative and provided additional evidence for the hydroxylation at C(11) on comparison with the data of **4**. The COSY-45° spectrum exhibited cross-peaks between H–C(11) (δ 3.95) and H–C(8) (δ 1.97). The configuration at C(11) was assigned to be (*R*) on the basis of NOE measurements: irradiation of H–C(11) (δ 3.95) resulted in a 12.4% enhancement of the β-oriented Me(13) (δ 0.98).

The HR-EI-MS of metabolite **8** exhibited M^+ at m/z 234.1619 ($C_{15}H_{22}O_2^+$), which is again 16 amu higher than M^+ of **4**. Another significant ion was at m/z 216.1525 ($C_{15}H_{20}O^+$) due to $[M-H_2O]^+$. An IR absorption band at 3421 cm^{-1} indicated the presence of an OH group. The structure of **8** was deduced to be (10*R*)-10-hydroxyisolongifolen-4-one.

The ^{1}H-NMR spectrum of **8** (*Table 2*) closely resembled that of **4** with an additional signal at δ 4.46 (*dd*, $J = 10.2, 2.9$ Hz), which could be assigned to H–C(10) on the basis of COSY-45° interactions of H–C(10) (δ 4.46) with H_a–C(9) (δ 1.85) and H_b–C(9) (δ 1.76). Metabolite **8** was further investigated by ^{13}C-NMR (*Table 3*), HMQC, and HMBC experiments. H–C(10) showed ^2J and ^3J heteronuclear couplings with C(1) (δ 59.8) and C(9) (δ 42.1), which further supported the position of the new OH group at C(10). The configuration at C(10) was concluded to be (*R*) on the basis of the NOE enhancement of the H–C(10) signal (7.8%) when the signal of the α-oriented Me(14) (δ 1.19) was irradiated.

The M^+ of compound **9** was observed at m/z 234.1610 ($C_{15}H_{22}O_2^+$) in the HR-EI-MS, containing one more O-atom than **4**, while the ion detected at m/z 216.1490 ($C_{15}H_{20}O^+$, $[M-H_2O]^+$) indicated the presence of an OH group, which was confirmed by the IR spectrum (3407 cm^{-1}). Metabolite **9** was characterized as (9*R*)-9-hydroxyisolongifolen-4-one. The configuration of the new OH group was assigned as α on the basis of a single-crystal X-ray analysis (*Fig. 2*).

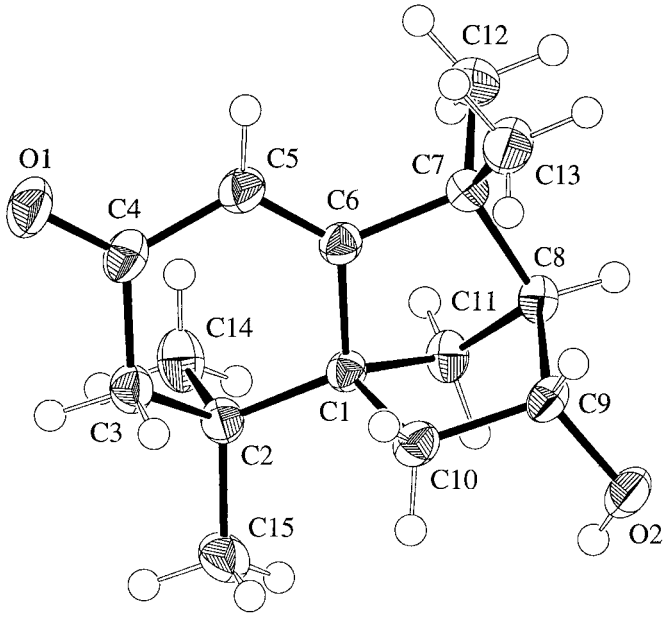


Fig. 2. *ORTEP Drawing of the X-ray crystal structure of* **9**[1])

The ^{1}H-NMR spectrum of **9** (*Table 2*) showed an additional CH *d* at δ 4.32 ($J = 6.5$ Hz) in comparison with **4**, corresponding to the C-atom resonating at δ 70.3 in the HMQC spectrum. The position of the new OH group was inferred as C(9) on the basis of COSY-45° interactions of H–C(9) (δ 4.32) with H–C(8) (δ 1.95) and HMBC interactions with C(10) (δ 40.2). H–C(8) also showed a ^2J heteronuclear interaction with C(9), which further supported the above assignment.

Experimental Part

1. *General*. Compounds **1**–**4** were purchased from *Sigma Aldrich*. TLC: silica gel precoated plates (*Merck*, PF_{254}; 20 × 20 cm, 0.25 mm). CC = Column chromatography. M.p.: *Büchi 535* melting-point apparatus. Optical rotations: *Jasco DIP-360* digital polarimeter. UV Spectra: $CHCl_3$ solns., *Hitachi U-3200* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: $CHCl_3$ solns., *FT-IR-8900* spectrophotometer; in cm^{-1}. ^1H- and ^{13}C-NMR Spectra: $CDCl_3$ solns.; *Bruker Avance-500-NMR* at 500 and 125 MHz, resp., 2D experiments with $CDCl_3$ solns. and the same instrument; chemical shifts δ in ppm rel. to $SiMe_4$ as internal standard, coupling constants J in Hz. EI-MS and HR-EI-MS: *Jeol JMS-600 H* mass spectrometer; in m/z (rel. %).

2. *Microorganisms and Culture Media*. Microbial cultures were obtained from either the *International Mycological Institute* (IMI), *American Type Culture Collection* (ATCC), or *Northern Regional Research Laboratories* (NRRL). All cultures were maintained on *Sabouraud*'s dextrose agar (SDA) and stored at 4° prior to use. The medium for *Fusarium lini* (NRRL 68751) was prepared by mixing the following ingredients into dist. H_2O (3.0 l): glucose (30.0 g), glycerol (30.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), and NaCl (15.0 g). The medium for *Aspergillus niger* (ATCC 10549) was prepared by adding the following chemicals into dist. H_2O (3.0 l): tartaric acid (105.0 g), sucrose (15.0 g), KH_2PO_4 (6.0 g), $MgSO_4 \cdot 2\ H_2O$ (3.0 g), NH_4NO_3 (6.0 g), and $Zn(OAc)_2$ (0.08 g), and 0.04N NaOH was used to increase the pH to 6.0. For *Rhizopus stolonifer* (ATCC 10404) the following ingredients were used (3.0 l, dist. H_2O): glucose (60.0 g), glycerol (30.0 g), peptone (30.0 g), yeast extract (8.0 g), and KH_2PO_4 (15.0 g), and the pH of the soln. was adjusted to 5.6 by adding a few drops of 0.04N NaOH. For *Cephalosporium aphidicola* (IMI 68689), the medium was prepared by mixing the following ingredients into dist. H_2O (3.0 l): glucose (150 g), KH_2PO_4 (3 g), KCl (3 g), $MgSO_4 \cdot 7\ H_2O$ (6 g), glycine (6 g), and *Gibberella* trace-element soln. (6 ml). The *Gibberella* trace-element soln. was prepared by mixing $Co(NO_3)_2 \cdot 6\ H_2O$ (0.01 g), $FeSO_4 \cdot 7\ H_2O$ (0.1 g), $CuSO_4 \cdot 5\ H_2O$ (0.1 g), $ZnSO_4 \cdot 7\ H_2O$ (0.161 g), $MnSO_4 \cdot 4\ H_2O$ (0.01 g), and ammonium molybdate (0.01 g) in dist. H_2O (100 ml).

7. *General Fermentation and Extraction Conditions*. The fermentation medium thus obtained was distributed among 30 250-ml flasks (100 ml in each) and autoclaved. The fermentation was carried out according to a standard two-stage protocol [12]. Compound **4** was dissolved in DMSO. The resulting clear soln. was evenly distributed among 30 flasks (20 mg/0.5 ml in each flask), containing 24-h-old stage-II cultures, and fermentation was carried out for further additional time on a rotatory shaker (200 rpm) at 29°. During the fermentation, aliquots from one culture were taken daily and analyzed by TLC to determine the degree of transformation of the substrate. In all experiments, one control flask without biomass (for checking substrate stability) and one flask without exogenous substrate (for checking endogenous metabolite) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with CH_2Cl_2 (1 l) and the filtrate extracted with CH_2Cl_2 (3 × 2 l). The combined org. extract was dried (Na_2SO_4) and evaporated, and the residue was analyzed by TLC. Control flasks were also harvested and compared with the test by TLC, to confirm the biotransformed products.

8. *Fermentation of Isolongifolen-4-one* (**4**) *with* Aspergillus niger (ATCC 10549). Compound **4** (600 mg), dissolved in DMSO (15 ml), was evenly distributed among 30 flasks containing stage-II cultures. Fermentation was stopped after 10 days, including control flasks. The org. metabolites were extracted from the medium and evaporated to afford a brown gum (2.7 gm). The crude residue was subjected to CC (petroleum ether/AcOEt gradient): **5** (27 mg; with petroleum ether/AcOEt 64 :36), **6** (78.1 mg; with petroleum ether/AcOEt 64 :36), and **7** (9.3 mg; with petroleum ether/AcOEt 59 :41).

*(7*R*)-12-Hydroxyisolongifolen-4-one* (= *(1*R,*2*S,*4a*R*)-1,2,3,4,5,6-Hexahydro-1-(hydroxymethyl)-1,5,5-trimethyl-7*H*-2,4a-methanonaphthalen-7-one*; **5**). White crystalline solid. M.p. 64°. $[\alpha]_D^{25} = -182$ ($c = 0.35$, $CHCl_3$). UV (MeOH): 248 (3.7). IR ($CHCl_3$): 3392, 2964, 1651, 772. ^1H-NMR ($CDCl_3$, 500 MHz): *Table 2*. ^{13}C-NMR ($CDCl_3$, 125 MHz): *Table 3*. EI-MS: 234 (66, M^+), 203 (49, $[M - CH_2OH]^+$), 176 (100), 161 (89), 147 (49), 119 (60), 91 (48), 55 (21). HR-EI-MS: 234.1617 (M^+, $C_{15}H_{22}O_2^+$; calc. 234.1620).

*(1*R,*2*S,*4a*R*)-1-[(Acetyloxy)methyl]-1,2,3,4,5,6-hexahydro-1,5,5-trimethyl-7*H*-2,4a-methanonaphthalen-7-one* (**10**). Compound **5** (24 mg) was treated with pyridine/Ac_2O 2 :1 and stirred for 6 h at r.t. The mixture was diluted with CH_2Cl_2 (20 ml) and the extract washed with 2% aq. $NaHCO_3$ soln. (20 ml) and 2% HCl soln. (20 ml), dried and evaporated: CC (Na_2SO_4), **10** (22.5 mg). Colorless oil. ^1H-NMR ($CDCl_3$, 500 MHz)[1]): 5.7 (br. *s*, H−C(5)); 3.94 (*d*, $J = 11.0$, H_a−C(12)); 3.81 (*d*, $J = 11.0$, H_b−C(12)); 2.04 (*s*, AcO); 2.13 (*t*, $J = 3.1$, H−C(8)); 1.17 (*s*, Me(13)); 1.01 (*s*, Me(14)); 0.94 (*s*, Me(15)). EI-MS: 276 (34, M^+), 216 (51, $[M - CH_2OAc]^+$), 201 (27), 161 (45), 160 (39), 117 (8.3), 83 (100). HR-EI-MS: 276.1722 (M^+, $C_{17}H_{24}O_3^+$; calc. 276.1725).

*(1*R,*2*S,*4a*R*)-1,2,3,4,5,6-Hexahydro-1-(methoxymethyl)-1,5,5-trimethyl-7*H*-2,4a-methanonaphthalen-7-one* (**11**). To **5** (21 mg, 0.09 mmol) in dried THF (10 ml), MeI (0.5 ml, 8 mmol) was added, followed by 60% NaH

dispersion in oil (3.6 mg, 0.09 mmol). The mixture, protected from the atmosphere with a drying tube, was stirred at r.t. for 1 h. The solvent was evaporated, the residue distributed between H_2O (10 ml) and CH_2Cl_2 (50 ml), and the CH_2Cl_2 layer washed twice with H_2O, dried (Na_2SO_4), and evaporated: **11** (18.7 mg). M.p. 50–51°. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.7 (*s*, H–C(5)); 3.24 (*d*, $J = 9.2$, H_a–C(12)); 3.0 (*d*, $J = 9.1$, H_b–C(12)); 3.31 (*s*, MeO); 2.26 (*s*, H–C(8), 1.17 (*s*, Me(13)); 1.03 (*s*, Me(14)); 0.96 (*s*, Me(15)). EI-MS: 248 (41, M^+), 220 (100), 205 (32), 173 (86), 147 (29), 119 (44), 91 (49), 55 (37). HR-EI-MS: 248.17104 (M^+, $C_{16}H_{24}O_2^+$; calc. 248.17162).

*(7*S*)-13-Hydroxyisolongifolen-4-one* (= *(1*S*,2*S*,4a*R*)-1,2,3,4,5,6-Hexahydro-1-(hydroxymethyl)-1,5,5-trimethyl-7*H*-2,4a-methanonaphthalen-7-one*; **6**). White crystals. M.p. 71°. $[\alpha]_D^{25} = -167$ ($c = 0.20$, $CHCl_3$). UV (MeOH): 247 (3.9). IR ($CHCl_3$): 3419, 2962, 1652, 1651. ^{1}H-NMR ($CDCl_3$, 500 MHz): *Table 2*. ^{13}C-NMR ($CDCl_3$, 125 MHz): *Table 3*. EI-MS: 234 (53, M^+), 203 (55, $[M - CH_2OH]^+$), 176 (100), 161 (97), 147 (14), 119 (17), 91 (66), 55 (33). HR-EI-MS: 234.1613 (M^+, $C_{15}H_{22}O_2$; calc. 234.1620).

*(1*S*,2*S*,4a*R*)-1-[(Acetyloxy)methyl]-1,2,3,4,5,6-hexahydro-1,5,5-trimethyl-7*H*-2,4a-methanonaphthalen-7-one* (**12**). As described for **10**, with **6** (17.3 mg): **12** (15.7 mg). Colorless oil. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.7 (br. *s*, H–C(5)); 4.02 (*d*, $J = 11.1$, H_a–C(13)); 3.95 (*d*, $J = 11.1$, H_b–C(13)); 2.04 (*s*, AcO); 2.17 (*t*, $J = 1.6$, H–C(8)); 1.13 (*s*, Me(12)); 1.02 (*s*, Me(14)); 0.96 (*s*, Me(15)). EI-MS: 276 (23, M^+), 216 (36, $[M - AcOH]^+$), 201 (27), 160 (35), 119 (9.1), 83 (100). HR-EI-MS: 276.17066 (M^+, $C_{17}H_{25}O_3^+$; calc. 276.17253).

*(1*S*,2*S*,4a*R*)-1,2,3,4,5,6-Hexahydro-1-(methoxymethyl)-1,5,5-trimethyl-7*H*-2,4a-methanonaphthalen-7-one* (**13**). As described for **11**, with **6** (24 mg, 0.10 mmol), NaH (4 mg, 0.1 mmol), THF (10 ml) and MeI (0.5 ml, 8 mmol) (addition in this order; 1 h): **13** (18.7 mg). White crystals. M.p. 63–65°. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.66 (*s*, H–C(5)); 3.26 (*d*, $J = 9.1$, H_a–C(13)); 3.18 (*d*, $J = 9.1$, H_b–C(13)); 3.33 (*s*, MeO); 2.15 (*d*, $J = 2.9$, H–C(8)); 1.15 (*s*, Me(12)); 1.03 (*s*, Me(14)); 0.97 (*s*, Me(15)). EI-MS: 248 (58, M^+), 220 (100), 205 (17), 173 (78), 147 (28), 119 (44), 91 (59), 55 (34). HR-EI-MS: 248.17033 (M^+, $C_{16}H_{24}O_2^+$; calc. 248.17762).

*(11*R*)-11-Hydroxyisolongifolen-4-one* (= *(2*S*,4a*R*,9*R*)-1,2,3,4,5,6-Hexahydro-9-hydroxy-1,1,5,5-tetramethyl-7*H*-2,4a-methanonaphthalen-7-one*; **7**). White crystals. M.p. 59–60°. $[\alpha]_D^{25} = -201$ ($c = 0.11$, $CHCl_3$). UV (MeOH): 242 (4.1). IR ($CHCl_3$): 3442, 2969, 1655. ^{1}H-NMR ($CDCl_3$, 500 MHz): *Table 2*. ^{13}C-NMR ($CDCl_3$, 125 MHz): *Table 3*. EI-MS: 234 (23, M^+), 216 (20, $[M - H_2O]^+$), 175 (100), 159 (28), 133 (30), 121 (69), 91 (56), 55 (58). HR-EI-MS: 234.1618 (M^+, $C_{15}H_{22}O_2^+$; calc. 234.16120).

9. *Fermentation of Isolongifolen-4-one* (**4**) *with* Fusarium lini (NRRL 68751). Compound **4** (600 mg), dissolved in 15 ml DMSO, was evenly distributed among 30 flasks containing 24-h-old biomass. Biotransformation was stopped after 7 days. The culture filtrate was extracted with CH_2Cl_2. CC of the org. extract (1.3 gm) yielded **8** (11.5 mg; with petroleum ether/AcOEt 75:25) and **9** (81.3 mg; with petroleum ether/AcOEt 72:28).

*(10*R*)-10-Hydroxyisolongifolen-4-one* (= *(2*R*,4*R*,4a*S*)-1,2,3,4,5,6-Hexahydro-4-hydroxy-1,1,5,5-Tetramethyl-7*H*-2,4a-methanonaphthalen-7-one*; **8**). White crystals. M.p. 59°. $[\alpha]_D^{25} = -181$ ($c = 0.11$, $CHCl_3$). UV (MeOH): 233 (4.9). IR ($CHCl_3$): 3421, 2958, 1652. ^{1}H-NMR ($CDCl_3$, 500 MHz): *Table 2*. ^{13}C-NMR ($CDCl_3$, 125 MHz): *Table 3*. EI-MS: 234 (23, M^+), 216 (20, $[M - H_2O]^+$), 175 (100), 159 (28), 133 (30), 121 (69), 91 (56), 55 (58). HR-EI-MS: 234.1619 (M^+, $C_{15}H_{22}O_2$; calc. 234.1620).

*(9*R*)-9-Hydroxyisolongifolen-4-one* (= *(2*S*,3*R*,4a*S*)-1,2,3,4,5,6-Hexahydro-3-hydroxy-1,1,5,5-tetramethyl-7*H*-2,4a-methanonaphthalen-7-one*; **9**): White crystal. M.p. 73–74°. $[\alpha]_D^{25} = -222$ ($c = 0.23$, $CHCl_3$). UV (MeOH): 244 (3.2). IR ($CHCl_3$): 3407, 2965, 1652. ^{1}H-NMR ($CDCl_3$, 500 MHz): *Table 2*. ^{13}C-NMR ($CDCl_3$, 125 MHz): *Table 3*. EI-MS: 234 (47, M^+), 216 (13, $[M - H_2O]^+$), 178 (51), 150 (71), 135 (70), 107 (35), 85 (100), 55 (42). HR-EI-MS: 234.1610 (M^+, $C_{15}H_{22}O_2^+$; calc. 234.1620).

*(2*S*,3*R*,4a*S*)-3-(Acetyloxy)-1,2,3,4,5,6-hexahydro-1,1,5,5-tetramethyl-7*H*-2,4a-methanonaphthalen-7-one* (**14**). As described for **10**, with **9** (25 mg) in pyridine/Ac_2O 2:1 (1 h): **14** (22.7 mg). Colorless oil. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.7 (br. *s*, H–C(5)); 5.08 (*d*, $J = 6.8$, H_β–C(9)); 2.02 (*s*, AcO); 2.07 (*s*, H–C(8)); 1.04 (*s*, Me(12)); 0.97 (*s*, Me(13)); 1.17 (*s*, Me(14)); 1.09 (*s*, Me(15)). EI-MS: 276 (23, M^+), 216 (96, $[M - AcOH]^+$), 201 (71, $[M - AcOH - Me]^+$), 173 (47), 160 (100), 133 (31), 91 (42), 55 (41). HR-EI-MS: 276.16624 (M^+, $C_{17}H_{24}O_3$; calc. 276.17253).

*(2*S*,3*R*,4a*S*)-1,2,3,4,5,6-Hexahydro-3-methoxy-1,1,5,5-tetramethyl-7*H*-2,4a-methanonaphthalen-7-one* (**15**). As described for **11**, with **9**: **15** (28.4 mg). White crystals. M.p. 66–67°. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.69 (br. *s*, H–C(5)); 3.73 (*d*, $J = 6.3$, H_β–C(9)); 3.27 (*s*, MeO); 2.03 (*s*, H–C(8)); 1.04 (*s*, Me(12)); 0.97 (*s*, Me(13)); 1.11 (*s*, Me(14)); 1.10 (*s*, Me(15)). EI-MS: 248 (31, M^+), 216 (17, $[M - MeOH]^+$), 201 (7), 192 (17), 175 (16), 160 (40), 119 (22), 99 (100), 55 (39). HR-EI-MS: 248.17258 (M^+, $C_{16}H_{24}O_2^+$; calc. 248.17762).

*(2*S*,3*R*,4a*S*)-3-[Ethoxy-1,2,3,4,5,6-hexahydro-1,1,5,5-tetramethyl-7*H*-2,4a-methanonaphthalen-7-one* (**16**): As described for **11**, with **9** (19.7 mg; 0.08 mmol), NaH (3.2 mg, 0.08 mmol), THF (6 ml), and EtI (0.5 ml, 6.1 mmol) (in this order; 1.5 h): **16** (18.1 mg). M.p. 57–59°. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.69 (br. *s*, H–C(5)); 3.83 (*d*, $J = 6.4$, H_β–C(9)); 3.43 (*m*, Me*C*H_2O); 1.18 (*t*, $J = 7.1$, *Me*CH_2O); 2.07 (*s*, H–C(8)); 1.05 (*s*, Me(12));

0.97 (*s*, Me(13)); 1.11 (*s*, Me(14)); 1.09 (*s*, Me(15)). EI-MS: 262 (49, M^+), 247 (27, $[M-Me]^+$), 216 (12), 192 (17), 175 (31), 160 (43), 99 (100), 55 (29). HR-EI-MS: 262.4668 (M^+, $C_{17}H_{26}O_2^+$; calc. 262.4617).

*(2*S*,3*R*,4a*S*)-1,2,3,4,5,6-Hexahydro-1,1,5,5-tetramethyl-3-(phenylmethoxy)-7*H*-2,4a-methanonaphthalen-7-one* (**17**). As described for **11**, with **9** (24 mg; 0.10 mmol), NaH (4 mg, 0.1 mmol), THF (10 ml), and benzyl bromide (0.5 ml, 0.21 mm; addition within 5 min) (in this order; reflux for 2 h): **17** (22.7 mg). Colorless oil. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.69 (br. *s*, H–C(5)); 7.31 (br. *s*, *Ph*CH_2O); 4.47 (*d*, $J=11.8$; PhCH_2O); 3.96 (*d*, $J=6.7$, H_β–C(9)); 2.1 (*s*, H–C(8)); 1.05 (*s*, Me(12)); 0.98 (*s*, Me(13)); 1.10 (*s*, Me(14)); 1.08 (*s*, Me(15)). EI-MS: 324 (3.4, M^+), 291 (2), 235 (1.6), 218 (5.7), 175 (55), 161 (6.3), 131 (12), 91 (100). HR-EI-MS: 324.21414 (M^+, $C_{22}H_{28}O_2^+$; calc. 324.20892).

*(2*S*,4a*S*)-1,2,5,6-Tetrahydro-1,1,5,5-tetramethyl-7*H*-2,4a-methanonaphthalene-3,7(4*H*)-dione* (**18**). Compound **9** (21.3 mg) was dissolved in $CHCl_3$ (5 ml), containing pyrdinium cholorochromate (1 equiv.). The mixture was stirred for 30 min at r.t. and filtered, the filtrate evaporated, and the residue purified by CC (silica gel, AcOEt/petroleum ether 3:6): **18** (19.1 mg). Colorless crystals. M.p. 42–43°. ^{1}H-NMR ($CDCl_3$, 500 MHz): 5.8 (*s*, H–C(5)); 2.4 (*s*, H–C(8)); 1.06 (*s*, Me(12)); 1.02 (*s*, Me(13)); 1.19 (*s*, Me(14)); 1.11 (*s*, Me(15)). EI-MS: 232 (3, M^+), 217 (17, $[M-Me]^+$), 190 (8.3), 175 (15), 161 (18), 133 (28), 83 (100), 55 (42). HR-EI-MS: 232.6214 (M^+, $C_{15}H_{20}O_2^+$; calc. 232.6297).

10. *Fermentation of Isolongifolen-4-one* (**4**) *with* Cephalosporium aphidicola (IMI 68689) *and* Rhizopus stolonifer (ATCC 10404). Biotransformation of **4** (600 mg/15 ml, for 3 l media) with *Cephalosporium aphidicola* (IMI 68689) yielded a single metabolite, **9** (51.8 mg), while *Rhizopus stolonifer* (ATCC 10404) gave metabolites **5** (91.4 mg) and **6** (69.5 mg), after 12 days of fermentation.

11. *Crystallographic Data of* **5**. A crystal of **5** suitable for X-ray-diffraction analysis was obtained by recrystallization from petroleum ether/CH_2Cl_2 3:1. A colorless block crystal with dimensions $0.19\times0.18\times0.11$ mm was selected for the crystallographic measurements. $C_{15}H_{22}O_2$: M_r 234.33; orthorhombic, $a=8.1839$ (2), $b=10.2182$ (3), $c=15.6343$ (5) Å, $V=1307.41$ (7) Å^3, space group = $P2_12_12_1$, $Z=4$, $D_{calc}=1.190$ mg/m^3, $F(000)=512$, Mo-K_α (λ 0.71069 Å). The unit-cell dimensions were determined by least-squares fit of 1715 reflections measured at 293 (2) K with Mo-K_α radiation and a *Nonius-Kappa-CCD* diffractometer. The intensity data within the θ range 4.0–27.5° were collected [13] at 173 (2) K. A total of 2983 reflections were recorded of which 2168 reflections were observed on the basis of $I>2\sigma(I)$. The structure was solved by direct methods [14] and expanded by *Fourier* techniques [15]. The structure ws refined by a full-matrix least-squares calculation on F^2 with the aid of the program SHELXL97 [16]. The final R and R_w factor were 0.041 and 0.110, resp. The figures were plotted with the aid of ORTEPII [17]. Crystallographic data for **5** and **9** (see *Sect. 12*) has been deposited with the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB21EZ, UK (fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

12. *Crystallographic Data of* **9**. As described in *Sect. 11:* Crystal of **9** from petroleum ether/CH_2Cl_2 5:1, dimensions $0.35\times0.20\times0.10$ mm. $C_{15}H_{22}O_2$: M_r 234.33; monoclinic, $a=9.2297$ (3), $b=8.2845$ (3), $c=9.4731$ (3) Å, $\beta=113.8470$ (18)°, $V=662.51$ (4) Å^3, space group = $P2_1$, $Z=2$, $D_{calc}=1.175$ mg/m^3, $F(000)=256$, Mo-K_α ($\lambda=0.71069$ Å); least-squares fit of 5541 reflections measured at 293 (2) K; θ range 4.1–27.5°; data collected [13] at 293 (2) K; of 2983 reflections recorded, 1298 reflections were observed on the basis of $I>2\sigma(I)$; final R and R_w, 0.041 and 0.110, resp.

13. *Enzyme-Inhibition Assay.* Tyrosinase-inhibition assays were performed in 96-well microplate formate with L-dopa (*Sigma Chemical Co.*, MO, USA) as substrate, the *SpectraMax-340* microplate reader (*Molecular Devices*, CA, USA), and the developed method described earlier [18]. All the compounds were dissolved in DMSO. The final solvent mixture was 2.5%. Briefly, 30 units of mushroom tyrosinase (28 nM purchased from *Sigma Chemical Co.*, MO, USA) was first preincubated with the compounds in 50 nM sodium phosphate buffer (pH 6.8) for 10 min at 25°. Then the L-dopa (0.5 mM) was added to the mixture, and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37°) due to the formation of the dopa-chrome for 10 min. The percent inhibition of the enzyme was calculated as follows, with a *MS-Excel®-2000* based (*Microsoft Corp.*, USA) program developed for this purpose:

$$\%\ \text{Inhibition} = [A_{blank} - A_{sample}/A_{blank}]\cdot 100$$

Therein A_{blank} is the absorbance for the blank and A_{sample} the absorbance for the samples. All the experiments were done at least in triplicate, and the results represent the mean ± s.e.m. (standard error of the mean). Kojic acid and L-mimosine (*Sigma Chem Co.*, MO, USA) were used as standards.

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