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MICROBIAL HYDROXYLATION OF SCLAREOL*

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Key Word Index—*Cunninghamella elegans*; Zygomycetes; sclareol; diterpene; microbial hydroxylation; phylogeny.

Abstract—Biotransformation of sclareol with different microorganisms led to 2α-, 3β- and 18-hydroxy-sclareol. *Cunninghamella elegans* additionally formed the hitherto unknown 19-hydroxy-sclareol. The phylogenetic position of a strain is mirrored in its ability to form these metabolites. The ability of fungi to convert this substrate is higher than that of the bacteria. Thus 2α-hydroxy-sclareol is only formed by zygomycotina and some deuteromycotina. The most active strains belong to the zygomycotina and basidiomycotina. Within the bacteria, Gram-positive bacteria are more active than Gram-negative ones.

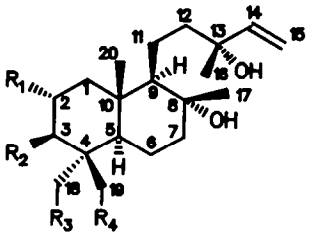
INTRODUCTION

I am interested in the study of the microbiological transformation of natural and synthetic terpenoids by strains of different phyla [1, 2]. These studies are mainly directed to preparing new hydroxylated synthetic intermediates from readily available precursors and to producing data which should allow a better pre-selection of suitable strains.

I selected sclareol (**1**) (Fig. 1), a labdane diterpene, as a substrate, since it can easily be isolated from the essential oil of *Salvia sclarea* L. (Labiatae) (clary sage oil). It is also produced by *Nicotiana* spp. and acts at the leaf surface as a fungal-growth regulator [3]. Technically it is used as a synthon for the preparation of a series of Ambra odorants in perfumery [4]. Some biotransformations with this substrate had already been reported, i.e. hydroxylation at the 3β- and 18-positions with *Cunninghamella* sp. NRRL 5695 [5] and at the 2α-position with *Septomyxa affinis* ATCC 6737 [6] and *Bacillus cereus*, beside glucoside conjugation [7]. A study directed to the microbial conversion of sclareol to a precursor of forskolin with *Mucor plumbeus* ATCC 4740 resulted in 6α-hydroxylation of the substrate [8]. In a patent, Farbood and coworkers claimed the degradation of sclareol to sclareolide with *Bensingtonia ciliata*, *Cryptococcus albidus* or *C. laurentii* in high yield and at high substrate concentration [9].

RESULTS AND DISCUSSION

Although sclareol is reported to suppress fungal growth [10, 11], I observed no retardation in the bio-



	R_1	R_2	R_3	R_4
1	H	H	H	H
2	OH	H	H	H
3	H	OH	H	H
4	H	H	OH	H
5	H	H	H	OH

Fig. 1. Sclareol **1** and its metabolites **2–5**.

transformations. Many strains were able to convert the substrate with fungi exhibiting a higher activity than bacteria. *Bacillus sphaericus* ATCC 13805, *Cunninghamella elegans* DSM 1908 and *Diplodia gossypina* ATCC 10936 were selected for preparative fermentation to produce the metabolites in sufficient amount for structure elucidation.

Bacillus sphaericus converted sclareol completely forming 3β-hydroxy-sclareol (**3**) (40% yield) and 18-hydroxy-sclareol (**4**) (10% yield), while *C. elegans* gave 2α-hydroxysclareol (**2**), 3β-hydroxysclareol (**3**) and 18-hydroxysclareol (**4**). These metabolites were identified by comparison of their NMR data with the published values. The least polar metabolite (**5**) however, could not be identified from literature data. The ^{1}H NMR spectrum

*Part IV in the series: 'Phylogeny and biotransformation'. For Part III see ref. [1].

showed only four singlets for methyl groups instead of five as in sclareol. Additionally, two doublets were seen at δ3.44 and 3.68 pointing to a hydroxymethyl group. This finding was confirmed by the ^{13}C NMR spectra where a triplet at δ64.8 was seen. The identity of the methyl group in sclareol which is hydroxylated in **5** could also be deduced from the ^{13}C NMR data. Thus comparison with the ^{13}C NMR spectrum of **5** with that of sclareol revealed that C-4 is deshielded (δ_C 33.2–38.4), while C-3 is shielded (δ_C 42.0–35.5) requiring a hydroxylation at one of the geminal methyl groups. With the assignment of the substrate in hand, we identified **5** as the endo-alcohol because the resonance of the adjacent methyl group was at δ_C 17.3. A high-field shift of δ48.2 is well in the range of the predicted γ-effect at C-18 of the hydroxy group. The shift change of δ16.1 (from δ33.4 to 17.3) that would have occurred if **5** was the exo-alcohol, is very unreasonable. The same arguments can be applied for the assignments of **4**.

Diplodia gossypina hydroxylated sclareol at the 2α-, 3β-, 18- and 19-positions.

The different phylogenetic groups of microorganisms show different metabolites. From Figs 2–5 it is obvious that 3β-hydroxylation is the most frequent reaction in the biotransformation of sclareol. 3β-Hydroxylation and 18-hydroxylation is seen with bacteria (Fig. 5), while 2α- and 19-hydroxylation are limited, at least in our screen, to fungi. The activities of the bacteria were lower than those of the fungi and within the bacteria the Gram-negative bacteria were the least active. The ability to form 2α-hydroxysclareol (**2**) was confined to the phyla Deuteromycotina and Zygomycotina while Basidiomycotina and Ascomycotina did not show this oxidation of the substrate.

A statistical survey of the biotransformation abilities of the individual phyla showed that there are considerable differences between them and that the Zygomycotina were the most active phylum in the biotransformation of sclareol, such an analysis is a valuable aid in the selection of the best strains for the biotransformation of this diterpene and may lead to a reduction of the screening effort.

Table 1. ^{13}C NMR data of compounds **1**, **4** and **5** ($CDCl_3$, 75.5 MHz)

C	1	4	5
1	39.7 —*	39.2 —	39.7 —
2	18.4 —	17.7 —	18.0 —
3	42.0 —	35.2 —	35.5 —
4	33.2 0	37.6 0	38.4 0
5	56.1 +	49.2 +	56.7 +
6	20.5	20.2	20.6 -
7	44.4 —	44.0 —	44.2 —
8	74.7 0	74.6 0	74.4 0
9	61.6 +	61.7 +	61.6 +
10	39.3 0	39.1 0	39.1 0
11	19.1 -	19.1 —	19.1 -
12	45.0 -	44.9 —	44.7 —
13	73.6 0	73.6 0	73.3 0
14	146.0 +	146.0 +	146.1 +
15	111.2 -	111.2 -	110.9 —
16	27.2 +	27.1 +	26.9 +
17	24.2 +	24.1 +	23.8 +
18	33.4 +	72.0 —	26.2 +
19	21.5 +	17.3 +	64.8 -
20	15.3 +	15.7 +	15.8 +

*Amplitude of signals in DEPT-135 spectrum (Me or CH = +; CH_2 = ; quat. C = 0).

EXPERIMENTAL

One hundred of the most active strains (40 bacteria and 60 fungi) were selected from our strain collection (Table 2). They were tested in a medium containing glucose (5 g l^{-1}), malt extract (5 g l^{-1}), peptone (2 g l^{-1}), yeast extract (5 g l^{-1}) and sclareol.

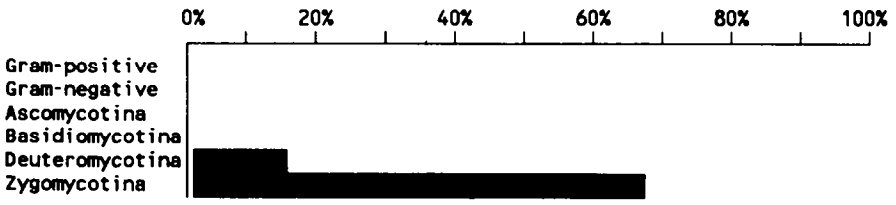


Fig. 2. Frequency of formation of **2**.

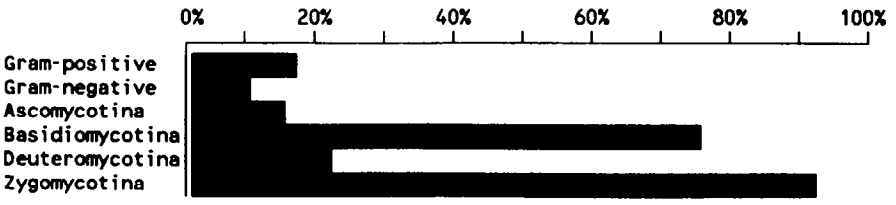


Fig. 3. Frequency of formation of **3**.

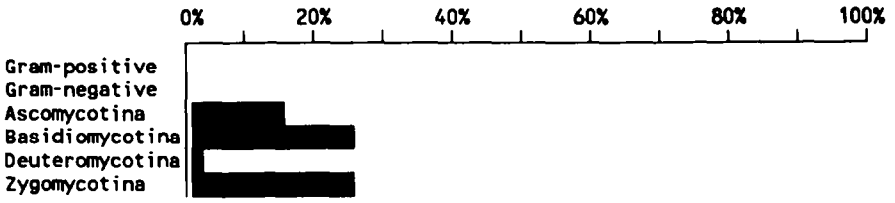


Fig. 4. Frequency of formation of **4**.

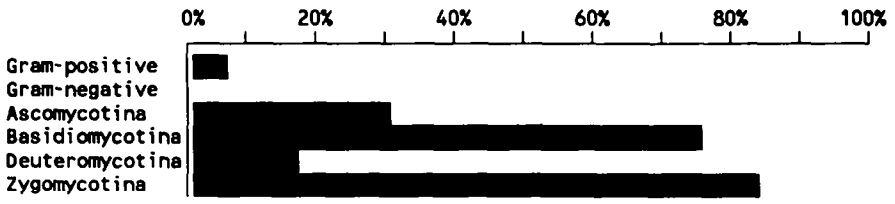


Fig. 5. Frequency of formation of **5**.

Starting 24 hr after substrate addition (0.5 $g l^{-1}$), samples were taken each day and analysed as follows. To 1 ml of culture broth 0.2 ml of EtOAc was added, shaken for 2 min, centrifuged and 10 μl of the extract subjected to HPLC (EtOAc). The spots were made visible by spraying with anisaldehyde–H_2SO_4 in HOAc and heating to 110° for 1 min. A video camera connected with a frame store board (SYNAPSE card) in a personal computer (1MB RAM, 80286 processor with 80287 coprocessor) and SW 2000 software (Ultraviolet Products Gel Analysis System, Cambridge, U.K.), was used to determine the R_f values of the biotransformation products and their intensity. Data management was done in a dBase file and a program was written for arrangement of the data for evaluation with the MULVA-4 program [12]. Basic statistics were performed with some other statistic programs. The individual spots were identified using reference substances, unknown products were isolated and their structures elucidated.

Extraction and purification. Culture medium and mycelia were separated by filtration and both were extracted (× 3) with EtOAc. The solvent was evapd and the crude extract sepd on silica-60 columns with a n-hexane–EtOAc gradient (changing from 9:1, to 0:1). When necessary the collected frs were further purified by prep. TLC.

Analysis. NMR: 400 MHz (^{1}H) and 75.5 MHz (^{13}C), $CDCl_3$ as solvent, and TMS as int. standard; MS: 70 eV; IR: $CHCl_3$; Mp: uncorr.

Bacillus sphaericus ATCC 13805 converted sclareol (20 mg) over a 72 hr period to **3** (4 mg) and **4** (1 mg).

Fermentation of sclareol (**1**) (200 mg) with *Cunninghamella elegans* DSM 1908 yielded, after 120 hr, **1** (81 mg), **3** (12 mg), **4** (20 mg), 13-epi-**4** (5 mg) and **5** (37 mg).

19-Hydroxy-sclareol (**5**). R_f 0.51 (EtOAc), crystals mp 115–116° IR: νcm^{-1}: 3373, 2964, 2932, 1458, 1387, 1035; ^{1}H NMR: 0.78 (3H, *s*, H-20), 0.94 (1H, *m*, H-3α), 0.97 (3H, *s*, H-18), 0.99 (1H, *m*, H-5), 1.15 (3H, *s*, H-17), 1.28 (3H, *s*, H-16), 1.5 (2H, *m*, H-2), 1.76 (1H, *m*, *H*-3β), 3.44 (1H, *d*, *J*

Table 2. Alphabetical list of strains used in this study

Absidia blakesleeana	ATCC	10148a
A. coerulea	ATCC	8990
Alcaligenes eutrophus	DSM	516
A. faecalis	DSM	30030
Amycolata autotrophica	DSM	535
Arthrobacter atrocyaneus	DSM	20127
A. oxydans	DSM	20119
A. petroleophagus	ATCC	21494
A. simplex	ATCC	13260
Aspergillus flavus	DSM	1959
A. niger	ATCC	9142
A. ochraceus	NRRL	405
A. terreus	DSM	62071
Bacillus cereus	DSM	508
B. megaterium	DSM	32
B. megaterium	DSM	333
B. megaterium	DSM	510
B. megaterium	DSM	1515
B. pumilus	DSM	27
B. sphaericus	ATCC	13805
Beauveria bassiana	ATCC	7159
Candida tropicalis	DSM	1346
Chaetomium cochliodes	ATCC	10195
C. globosum	DSM	62109
Coriolus versicolor	IFO	4937
Corynebacterium equi		
C. sp.	ATCC	15570
Corynespora cassiicola	DSM	62474
Cunninghamella blakesleeana	ATCC	8983
C. elegans	DSM	1908
Curvularia affinis	DSM	63274
C. fallax	DSM	63169
C. pallescens	DSM	62482
Diplodia gossypina	ATCC	10936
Fusarium ciliatum	DSM	62172
F. ciliatum	DSM	879
F. coeruleum	DSM	62178
F. concolor	DSM	62179
F. dimerum	DSM	62197

Table 2. *Continued*

F. fujikuroi	DSM	893
F. graminearum	DSM	1095
F. graminearum	DSM	62722
F. inflexum	DSM	63203
F. lateritium	DSM	62244
F. oxysporum	ATCC	9593
F. oxysporum	DSM	62291
F. oxysporum f. *aechmeae*	DSM	62297
F. oxysporum f. sp. *pisi*	ATCC	9991
F. oxysporum f. sp. *vasinfectum*	ATCC	7808
F. reticulatum	DSM	62719
F. roseum	DSM	3019
F. solani	DSM	62413
F. tabacinum	DSM	2125
F. tricinctum	DSM	62446
F. verticillioides	DSM	764
F. verticillioides	DSM	840
Gliocladium roseum	ATCC	8684
Glomerella cingulata	ATCC	10529
Mortierella isabellina	DSM	63355
Mucor circinelloides	CBS	394.68
M. circinelloides f. *lusitanicus*	CBS	277.49
M. indicus	CBS	226.29
Mycobacterium fortuitum	ATCC	6842
M. phlei	DSM	2354
M. smegmatis	DSM	43061
M. smegmatis	*DSM*	43299
M. sp.	DSM	43293
Nocardia calcarea	DSM	43188
N. gardneri	DSM	43020
N. sp.	DSM	43130
Ophiostoma picea		
Pellicularia filamentosa	IFO	6259
Penicillium camemberti	ATCC	4845
P. digitatum	DSM	62840
P. diversum	CBS	32048
P. verruculosum	ATCC	10483
Polyporus eucalyptorum	CBS	30739
Pseudomonas cepacia	DSM	50180
P. fluorescens	ATCC	948
P. fluorescens	DSM	84
P. lapsa	DSM	50274
P. oleovorans	ATCC	13474
P. putida	DSM	291
Rhizopus oryzae	ATCC	11145
R. oryzae	CBS	128.08
R. stolonifer	ATCC	10404
Rhodococcus erythropolis	DSM	43274
R. rhodochrous	DSM	43002
R. rubropertinctus	DSM	43197
Serratia liquefaciens	DSM	30064
S. marcescens	DSM	1608
Streptomyces albofaciens	DSM	40268
S. bacillaris	DSM	40598
S. bikiniensis	IFO	13350
S. griseus	ATCC	21897
S. parvus	IFO	3388
Syncephalastrum racemosum	DSM	859
Trametes versicolor	DSM	1977
Trichoderma viride	DSM	63065
Yarrowia lipolytica	IFO	1542

= 11 Hz, H-19), 3.68 (1H, *d*, *J* = 11 Hz, H-19'), 5.04 (1H, *dd*, *J* = 10, 1 Hz, H-15'), 5.21 (1H, *dd*, *J* = 17, 1 Hz, H-15), 5.93 (1H, *dd*, *J* = 17, 10 Hz, H-14). MS *m/z*: 306.2559 (306.2559 calc. for $C_{20}H_{34}O_2$) $[M-H_2O]^+$ (20%), 291 (32), 175 (40), 149 (76), 95 (71), 43 (100);

$$[\alpha]^{27} = \frac{\begin{matrix}589\text{nm} & 578\text{nm} & 546\text{nm} & 436\text{nm} & 365\text{nm}\end{matrix}}{\begin{matrix}-6.0^\circ & -6.1^\circ & -6.4^\circ & -9.6^\circ & -17.0^\circ\end{matrix}} (c\ 1.00).$$

Biotransformation of sclareol (10 mg) with *Diplodia gossypina* ATCC 10936 resulted, after 96 hr, in **2** (2 mg), **3** (2 mg), **4** (2 mg) and **5** (2 mg).

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REFERENCES

1. Abraham, W.-R. (1994) *World J. Microbiol. Biotechnol.* (in press).
2. Abraham, W.-R. (1993) *World J. Microbiol. Biotechnol.* **9**, 319.
3. Kennedy, B. S., Nielsen, M. T., Severson, R. F., Sisson, V. A., Stephenson, M. K. and Jackson, D. M. (1992) *J. Chem. Ecol.* **18**, 1467.
4. Decorzant, R., Vial, C., Näf, F. and Whitesides, G. M. (1987) *Tetrahedron* **24**, 1871.
5. Kouzi, S. A. and McChesney, J. D. (1991) *J. Nat. Prod.* **54**, 483.
6. Kouzi, S. A. and McChesney, J. D. (1990) *Helv. Chim. Acta* **73**, 2157.
7. Kouzi, S. A. and McChesney, J. D. (1991) *Xenobiotica* **21**, 1311.
8. Aranda, G., Lallemand, J.-Y., Hammoumi, A. and Azerad, R. (1991) *Tetrahedron Letters* **32**, 1783.
9. Farbood, M. I., Morris, J. A. and Downey, A. E. *United States Patent* No. 4, 970, 163, Nov. 13, 1990.
10. Bailey, J. A., Vincent, G. G. and Burden, R. S. (1974) *J. Gen. Microbiol.* **85**, 57.
11. Bailey, J. A., Carter, G. A., Burden, R. S. and Wain, R. L. (1975) *Nature* **255**, 328.
12. Wildi, O. and Orloci, L. (1990) *MULVA*-4, a package for multivariate analysis of vegetation data. SPB Academic Publishing, The Hague.