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Biotransformation and Phylogeny

VI. Microbial Oxidation of Aristolenepoxide to Phytotoxins[1]

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Main metabolites of the microbial oxidation of (6R)-aristolenepoxide are 5α-, 9β-, 10β-, and 15-hydroxy-aristolenepoxide. Aristolene-7,14-oxide-6α-ol was also formed. Some of them showed modest phytotoxic activity in the cress test, where 9β-hydroxy-aristolenepoxide is the most active one. The actvity of the strains mirrored their phylogenetic positions. Bacteria were most active in oxidizing 15-hydroxy-aristolenepoxide to the aldehyde, while fungi, especially Basidiomycotina, were more active in the hydroxylation of the substrate than bacteria.

INTRODUCTION

We have been interested in the biotransformation of terpenoids with microorganisms for many years (*1*). Terpenes are widespread in nature and possess a multitude of biological activities. Some of the lower terpenoids, mostly hydrocarbons and alcohols, are available in larger quantities from essential oils at low to moderate prices. The idea is attractive to oxidize these compounds to biologically active natural products or their synthons. Because of the low functionality of these starting materials, chemical transformations are hampered. The use of microorganisms for this purpose is gaining more and more interest. A review on biotransformations of sesquiterpenoids, although incomplete, appeared recently (*2*).

In continuation of our studies on microbial oxidation of sesquiterpenoids we concentrated our efforts on the hydroxylation of tricyclic sesquiterpenes with an aristolane skeleton. Such a type of oxidation is chemically difficult because of (i) nonactivated carbon atoms and (ii) instability of the cyclopropane system compared to larger rings due to its strained bonds. These difficulties are typical of chemical transformations on such compounds but microorganisms can oxidize unreactive carbons under mild conditions with high regio- and stereoselectivity. Because of low yields in the biotransformation of aristolene (*3*) we oxidized the hydrocarbon to aristolenepoxide. This compound turned out to be a good substrate for most strains.

Dedicated to Professor A. Baerheim Swendsen on the occasion of his 70th birthday and to Professor K.-H. Kubeczka on the occasion of his 60th birthday.

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RESULTS AND DISCUSSION

To facilitate the structure elucidation of the expected metabolites the ^1H and ^{13}C NMR data of **1** were assigned. 2-H, 4-H, 6-H, and 12-H could immediately be assigned by their chemical shift and coupling pattern. COSY-45 2D-NMR spectra led to the assignment of the methylene protons and 11-H. Because a long range coupling was observed between the methyl groups at δ_H 1.04 and 1.00 these signals belong to the geminal methyl groups leaving the remaining methyl singlet for 13-H (Table 1). A $^{13}C/^1H$ two-dimensional NMR (HETERO-COSY) brought the assignments of carbons in the ^{13}C NMR (Table 2). The highfield resonance at δ_C 18.2 of the geminal methyl groups was assigned to the endo-methyl group following the empirical rule obtained from the ^{13}C NMR data of related sesquiterpenes (*4*).

In the biotransformation most strains formed a compound which displayed an absorption of a hydroxy group in the IR spectrum. The ^1H NMR spectrum showed only three instead of the original four methyl groups and an AB quartet at δ_H 3.39 and 3.26 pointing to a hydroxymethyl group. This assumption is confirmed by the ^{13}C NMR spectrum which revealed a triplet at δ_C 73.8. 2D ^1H NMR spectra and the assignment and comparison of the ^{13}C NMR data with **1** led to the structure of 15-hydroxy-aristolenepoxide **10** (Fig. 1). The exo-position of the hydroxymethyl group is derived from the shift of the geminal methyl group which is shielded from δ_C 18.2 to 13.1 which is well within the range of the predicted γ-effect of the hydroxy group at C-15. Only a few strains, mainly bacteria, were able to oxidize this compound to the aldehyde **2** (Fig. 2).

The search for the endo-epimere of **10** was unsuccesful. Instead, an isomeric compound was found, formed in larger amount by *Cunninghamella spp.* The mass spectrum showed a molecular ion with the composition $C_{15}H_{24}O_2$. In the ^1H NMR spectrum again only three methyl groups were seen and the fourth one is oxidized as judged from the AB quartet at δ_H 3.92. The 6-H was deshielded to δ_H 3.64 and displayed larger coupling constants (J = 10, 4.5 Hz) than the epoxide-proton at **1.** Such coupling constants are only in agreement with an axial proton in a cyclohexane ring. The remaining protons could be identified with the aid of the COSY-45 2D-NMR spectra. The δ_C of 26.5 of C-15 indicated an endo-oxidation at the geminal methyl groups. Here C-15 is shielded from δ_C 29.7 to 26.5, which is in perfect agreement with the data observed for compound **10.** The molecular formula requires two oxygen while the ^{13}C NMR spectrum demands three carbons connected to oxygen. This is only possible if one oxygen forms an ether. Since there is no epoxide anymore as judged from the coupling constant of 6-H, C-14 is bearing the etheroxygen. This oxygen could only be connected to C-7 because 6-H must be located in a cyclohexane in chair conformation otherwise the coupling constants of this proton could not be explained. Taking all these informations together the structure **11** results for this compound. Its formation can be explained by a hydroxylation of C-14 resulting in an alcohol which opened intramoleculary the epoxide to yield **11.**

Beside these compounds some secondary alcohols were formed. The structure of **3, 5, 6,** and **7** could be deduced from the COSY 45 2D NMR and ^{13}C NMR data using the assignment of the spectrum of **1** and empirical rules of ^{13}C NMR shifts

TABLE 1
^{1}H NMR Data of **1–11** ($CDCl_3$, 400 MHz)

	1	**2**	**3**	**4**	**5**	**6**
2-H	0.24 d	1.17 d	0.25 d	0.30 d	0.51 d	0.37 d
4-H	0.58 ddd	1.48 m	0.58 ddd	0.55 dd	0.67 ddd	0.65 dd
5-H	2.24 dd	2.39 dd	2.24 ddd	3.91 d	2.04 ddd	3.98 d
5′-H	1.74 ddd	1.81 ddd	1.80 ddd	—	1.71 m	—
6-H	2.91 m	2.98 d	2.98 dd	3.02 dd	3.84 dd	3.08 dd
8-H	1.97 ddd	1.96 ddd	2.11 dd	2.04 dd	4.21 dd	1.97 m
8′-H	1.19 dm	1.71 m	1.35 dd	1.29 ddd	—	1.23 m
9-H	1.69 m	1.45 m	4.12 dddd	4.03 dddd	2.18 m	1.73 m
9′-H	1.29 m	1.3 m	—	—	1.59 m	1.26 m
10-H	1.43 m	1.45 m	1.54 m	1.46 m	1.74 m	1.45 m
10′-H	1.29 m	1.3 m			1.31 m	1.26 m
11-H	1.75 m	1.66 m	2.30 m	2.23 m	2.20 m	1.75 m
12-H	1.01 d	0.90 d	1.00 d	0.90 d	0.88 d	1.00 d
13-H	1.12 s	1.22 s	1.10 s	0.98 s	1.14 s	1.13 s
14-H	1.04 s	1.22 s	1.05 s	1.03 s	1.01 s	1.07 s
15-H	1.00 s	8.65 s	1.04 s	1.00 s	1.28 s	1.01 s
	7	**8**	**9**	**10**	**11**	
2-H	0.23 d	5.41 d	0.38 d	0.37 d	0.95 d	
4-H	0.58 ddd	2.24 dd	0.73 ddd	0.68 ddd	0.75 ddd	
5α-H	2.25 dd	1.84 m	2.30 ddd	2.23 ddd	2.58 ddd	
5β-H	1.76 ddd	1.71 m	1.78 ddd	1.73 ddd	1.98 ddd	
6-H	2.94 d br	3.60 dd	2.98 dd	2.95 m	3.64 dd	
8α-H	2.08 m	1.84 m	2.08 m	1.93 ddd	1.84 ddd	
8β-H	1.31 m	1.04 m	1.33 m	1.17 m	1.12 dm	
9α-H	1.99 m	1.75 m	2.00 m	1.67 m	1.45 dm	
9β-H	1.31 m	1.5 m	1.33 m	1.23 m	1.68 ddddd	
10α-H	3.54 ddd	1.5 m	3.55 ddd	1.41 m	1.19 dddd	
10β-H	—	1.5 m	—	1.20 m	1.38 dm	
11-H	1.67 dq	2.54 m	1.66 dq	1.72 m	1.85 m	
12-H	1.15 d	1.18 d	1.17 d	0.97 d	0.86 d	
13-H	1.11 s	1.08 s	1.16 s	1.14 s	1.00 s	
14-H	1.05 s	1.26 s	1.12 s	1.11 s	3.92 ABq	
15-H	1.00 s	1.24 s	3.38 d	3.39 d	0.96 s	
15′-H			3.30 d	3.26 d		

Note. J(Hz): **1, 2:** 2,4 = 4,5 = 10; 5,5′ = 16; 5′,6 = 2; 8,8′ = 14; 8,9 = 12; 8,9′ = 4; 11,12 = 7. **3:** 2,4 = 4,5 = 10; 4,5′ = 1; 5,5′ = 16; 5,6 = 1; 5′,6 = 2; 8,8′ = 16; 8,9 = 3; 8′,9 = 4; 9,10 = 9,10′ = 4; 11,12 = 6.8. **4:** 2,4 = 10; 4,5 = 2; 4,6 > 0; 5,6 = 2; 8,8′ = 16; 8,9 = 3; 8′,9 = 4; 9,10 = 9,10′ = 4; 11,12 = 7. **5:** 2,4 = 4,5 = 10; 4,5′ = 6; 5,5′ = 16; 5,6 = 5′,6 = 3; 8,9 = 8,9′ = 6; 11,12 = 7. **6:** 2,4 = 10; 4,5 = 2; 4,6 > 0; 5,6 = 2; 11,12 = 7. **7:** 2,4 = 4,5 = 10; 4,5′ = 2; 5,5′ = 16; 5′,6 = 2; 9,10 = 4; 9′,10 = 10,11 = 10; 11,12 = 7. **8:** 2,4 = 4; 4,5 = 3; 4,5′ = 8; 5,6 = 5; 5′,6 = 12; 11,12 = 7. **9:** 2,4 = 4,5 = 9.5; 4,5′ = 2; 5,5′ = 16.5; 5,6 > 0; 5′,6 = 2; 9,10 = 4.3; 9′,10 = 10,11 = 10.5; 11,12 = 6.8; 15,15′ = 10.8. **10:** 2,4 = 4,5 = 10; 4,5′ = 1; 5,5′ = 16; 5,6 = 1; 5′,6 = 2; 8,8′ = 13; 8,9 = 13; 8,9′ = 3; 11,12 = 7; 15,15′ = 11. **11:** 2,4 = 9; 4,5α = 1.7; 4,5β = 5; 5α,5β = 15.5; 5α,6 = 10; 5β,6 = 4.5; 8α,8β = 13; 8α,9α = 4.5; 8α,9β = 13; 8β,9α = 3; 8β,9β = 4; 9α,9β = 13; 9α,10α = 4.5; 9α,10β = 3; 9β,10α = 13; 9β,10β = 4; 10α,10β = 13; 10α,11 = 4; 11,12 = 7; 14, 14′ = 10.5.

TABLE 2
^{13}C-NMR Data of **1–4, 6, 7, 10,** and **11** ($CDCl_3$, 75.5 MHz)

	1	**2**	**3**	**4**	**6**	**7**	**10**	**11**
C-1	35.3 0[a]	35.2 0	34.8 0	35.3 0	35.7 0	n. d.	n. d.	34.7 0
C-2	29.5 +	29.9 +	29.5 +	30.5 +	30.7 +	29.6 +	26.7 +	23.5 +
C-3	17.5 0	33.9 0	17.4 0	16.2 0	16.1 0	n. d.	24.0 0	16.6 0
C-4	16.2 +	16.1 +	16.0 +[b]	25.1 +	25.7 +	15.8 +	13.5 +[c]	15.2 +
C-5	20.3 −	19.7 −	20.0 +	66.7 +	66.6 +	20.1 −	19.9 −	28.3 −
C-6	63.8 +	63.0 +	64.2 +	63.9 +	64.3 +	63.4 +	63.7 +	72.8 +
C-7	63.2 0	62.9 0	61.7 0	65.5 0	67.5 0	n. d.	63.4 0	75.5 0
C-8	32.7 −	32.4 −	39.0 −	38.7 −	32.5 −	30.5 −	32.7 −	29.4 −
C-9	24.5 −	24.0 −	66.1 +	65.8 +	24.2 −	33.5 −	24.3 −	20.6 −
C-10	30.1 −	29.8 −	36.6 −	36.5 −	29.9 −	71.8 +	30.1 −	30.1 −
C-11	37.4 +	36.9 +	29.2 +	29.3 +	37.5 +	44.4 +	37.2 +	36.4 +
C-12	16.5 +	17.5 +	15.9 +[b]	15.9 +	16.5 +	12.2 +	16.4 +	15.9 +
C-13	19.7 +	19.2 +	18.9 +	18.8 +	19.4 +	21.0 +	19.5 +	19.7 +
C-14	18.2 +	10.1 +	17.6 +	17.4 +	17.9 +	17.9+	13.1 +[c]	64.5 −
C-15	29.7 +	202.5 +	29.8 +	29.8 +	29.4 +	29.6 +	73.8 −	26.5 +

[a] Amplitude of signals in DEPT-135 spectrum (CH_3 or CH = +; CH_2 = −; quat. C = 0).
[b,c] Assignment may be interchanged.

caused by a hydroxy group. For all these compounds the configuration at the hydroxy group could be deduced from the coupling constants of the proton adjacent to the hydroxy moiety. Some of these alcohols were further hydroxylated by some strains yielding the diols **4** and **9** (Table 3). The formation of 5α-hydroxy- and 8α-hydroxy-aristolene-epoxide **5** and **6** is an interesting result because both hydroxy groups were introduced into the molecule syn to the epoxide. This requires an orientation of the epoxide toward the active center of the monooxygenase leaving no room for a separate fixation of the substrate via the epoxide.

Only one metabolite displayed resonances of sp^2-configurated carbons in the ^{13}C NMR spectrum indicating a rearrangement of the aristolane skeleton. The connectivity pattern derived from the 2D NMR spectrum led to compound **8** with the selinane skeleton. **8** is the 9-hydroxy derivative of rosifoliol, a sesquiterpene isolated from *Rubus rosifolius* (*5*, *6*). The formation of this metabolite represents some sort of a retro-biosynthesis since selinanes are the precursor of eremophilanes and aristolanes. The mechanism of the formation of **8** is not exactly known but a protonation, subsequent migration of the 13-methyl group from C-1 to C-7 and the fragmentation of the cyclopropane ring seems probable.

Some of these metabolites display modest activity in the cress phytotoxicity test. 9β-Hydroxy-aristolenepoxide **3** was the most active compound which inhibits the growth of the epicotyls with an ED_{50} of 760 μM, while the growth of the roots were inhibited with an ED_{50} of 850 μM. 5α-Hydroxy-aristolenepoxide **6** was not as active as **3,** giving ED_{50}s of 2.2 mM (epicotyl) and 1.5 mM (roots). 5α,9β-Dihydroxy-aristolenepoxide **4** combining the hydroxylation sites of **3** and **6** showed almost no activity. The phytotoxins **3** and **6** were almost exclusively formed by fungi. The only exception in our screen was *Streptomyces albofaciens* DSM 40268 (Table 4).

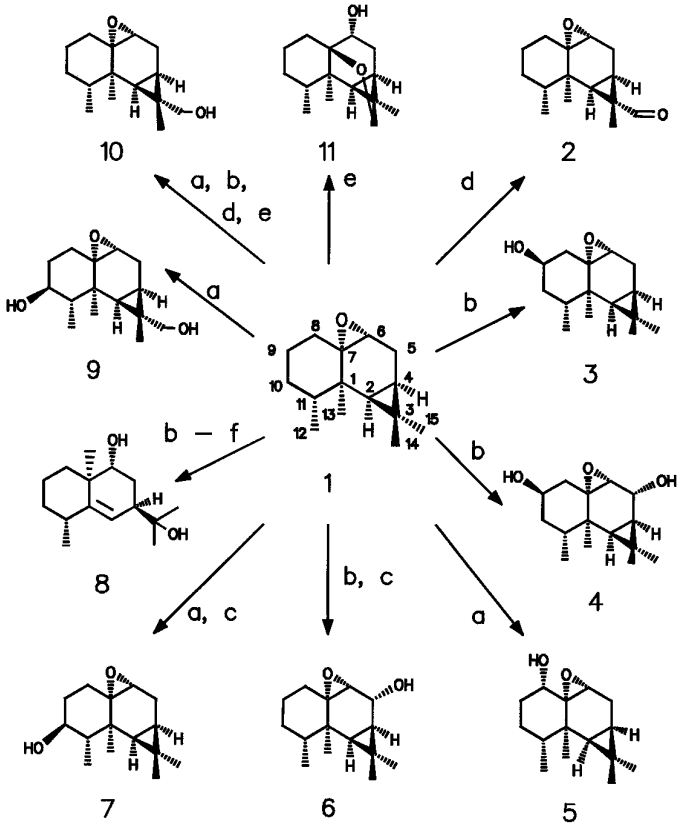

FIG. 1. Biotransformation products of aristolenepoxide **1** with a, *Botryosphaeria rhodina* ATCC 10936; b, *Curvularia fallax* DSM 63169; c, *C. pallescens* DSM 62482; d, *Aspergillus niger* ATCC 9142; e, *Cunninghamella elegans* DSM 1908; f, *Nocardia gardneri* DSM 43020.

EXPERIMENTAL

One hundred of the most active strains (40 bacteria and 60 fungi) were selected from our strain collection. They were tested in a medium containing glucose (5 g/liter), malt extract (5 g/liter), peptone (2 g/liter), and yeast extract (5 g/liter) with the substrate.

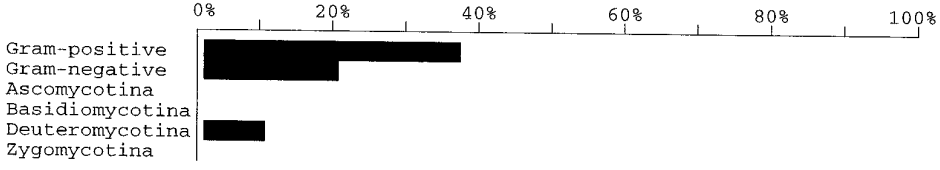

FIG. 2. Frequency of formation of aristolene-6α,7α-epoxide-15-al **2.**