

Biotransformation of Citral by *Botrytis cinerea*

Pascal Brunerie

Centre de Recherche, Pernod-Ricard, F-94015 Créteil, France

Irmgard Benda

Bayerische Landesanstalt für Weinbau und Gartenbau, D-8700 Würzburg

Gudrun Bock and Peter Schreier

Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, D-8700 Würzburg, Bundesrepublik Deutschland

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Biotransformation of citral (**1**) was studied with four strains of *Botrytis cinerea* using grape must (A), a synthetic medium (B) and mixtures of A and B. Whereas in A complete metabolization of **1** without evidence of any volatile product was observed, in B nerol (**2**) and geraniol (**3**) were found as predominant volatile bioconversion products; in minor amounts (*E,Z*)-2,6-dimethylocta-2,6-dien-1,8-diol (**4**) and (*E,E*)-2,6-dimethylocta-2,6-dien-1,8-diol (**5**), 2-methyl-2-hepten-6-one (**6**), 2-methyl-2-hepten-6-ol (**7**), 2-methyl-2-hepten-6-one-1-ol (**8**) and 2-methyl- γ -butyrolactone (**9**) were identified. Using small amounts of A in B (1:700 to 5:700) low yields of **2** and **3** were obtained, whereas the quantities of **4**, **5**, **6** and **8** increased. Quantitatively, the results were strongly dependent on the strains used. The bioconversion products were all identified by capillary gas chromatography (HRGC) and coupled HRGC techniques, *i.e.* -mass spectrometry (HRGC-MS) and -Fourier transform infrared spectroscopy (HRGC-FTIR).

Introduction

In the past, *Botrytis cinerea* has been recognized to be responsible for the degradation of terpenes observed in wines made from botrytized grapes [1, 2]. Recently, in bioconversion studies performed with linalool [3, 4] and citronellol [5] the first structural elucidation of biotransformation products formed by *B. cinerea* from terpene alcohols was achieved. In these investigations ω -hydroxylation has been found to be one of the predominant metabolic steps. Additionally, C₁ oxidation and, in part, hydrogenation of double bonds have been observed. Continuing our work on microbial transformation of terpenes, we also studied citral (**1**), which consists of the geometric isomers neral and geranial [6]. This α,β -unsaturated terpene aldehyde is highly important in perfumery and aroma compositions such as citrus flavours as well as starting material in the synthesis of ionones [7].

Materials and Methods

Botrytis cinerea strains

The *B. cinerea* strains 5899/4, 5901/2, 5882/1 and 5909/1, used in this study, were obtained from the collection of the Bayerische Landesanstalt für Weinbau und Gartenbau, Würzburg. From the original cultures, a part was transferred to malt agar slants and incubated at 25 °C for 7 days.

Media and incubation conditions

- Grape must. The sugar and acid content of the grape must (cultivar, Müller-Thurgau) used were adjusted to 200 g/l and 8.5 g/l (pH 3.5), respectively.
- Synthetic medium. The medium contained (per l): NaNO₃ 3 g; K₂HPO₄ 1 g; MgSO₄·7H₂O 0.5 g; KCl 0.5 g and FeSO₄ 0.01 g. The pH was adjusted to 3.5 using 1 N HCl.
- Synthetic medium/grape must. Mixtures of 700:1 to 700:5 were used.

The medium (a, b, c) (700 ml) was filled into 1 l-Erlenmeyer flasks and sterilized (30 min at 110 °C). After addition of 50 mg/l citral (**1**) (in 1 ml ethanol) each flask was inoculated with a pure *B. cinerea* strain and incubated at 25 °C for 2 weeks. The mycelium was removed by filtration and the solutions

Reprint requests to Prof. Dr. P. Schreier.

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analyzed by capillary gas chromatography (HRGC), capillary gas chromatography-mass spectrometry (HRGC-MS) and capillary gas chromatography-Fourier transform infrared spectroscopy (HRGC-FTIR) after extractive sample preparation. In the same manner, blank tests without *B. cinerea* incubation and without **1** were carried out.

Isolation of biotransformation products

After addition of internal standards (2-methyl-1-pentanol for the neutral and 2,2-dimethylpentanoic acid for the acidic fraction; both 0.50 mg/l) to the filtrate of the above-mentioned untreated and botrytized media solvent extraction was carried out using a pentane-dichloromethane mixture (2:1) [8]. Acids were removed from the extracts by separation with 5% NaHCO₃ solution (3 × 50 ml). The organic phase containing the neutral biotransformation products was carefully concentrated to 1 ml using a Vigreux column (45 °C) for subsequent HRGC, HRGC-MS and HRGC-FTIR analysis. The alkaline aqueous phase (acidic fraction) was acidified to pH 1–2 using 5 N HCl, extracted with diethyl ether (3 × 50 ml), methylated with CH₂N₂, and analyzed in the same manner as the neutral fraction.

Capillary gas chromatography (HRGC)

Instrument: Carlo Erba Fractovap 4160 fitted with a flame ionization detector (FID) and an air-cooled on-column injector. Column: J & W DB-Wax (30 m × 0.32 mm i.d.; d.f. = 0.25 µm) fused silica capillary, connected with a 2 m uncoated fused silica precolumn als "retention gap". On-column injection was used. The temperature program was isothermal for 3 min at 50 °C, then 50 °C to 240 °C at 5 °C/min. The flow rates for the carrier gas were 2 ml/min for He, for the make-up gas 30 ml/min N₂ as well as for the detector gases 30 ml/min H₂ and 300 ml/min air, respectively. The detector temperature was kept at 220 °C. Volumes of 0.5 µl were injected.

Results of qualitative analyses were verified by comparison of HRGC retention (*R*_t), mass spectral and vapour phase FTIR data with those of authentic reference substances. Quantitative determinations were carried out by standard controlled calculations using a Hewlett Packard 3388 A laboratory data system without consideration of extraction yields (calibration factors for all compounds, *F* = 1.00).

Capillary gas chromatography-mass spectrometry (HRGC-MS)

Instrument: Finnigan MAT 44 quadrupole mass spectrometer coupled by an open-split interface with a Varian Aerograph 1440 equipped with a water-cooled on-column injector. A J & W DB-Wax (30 m × 0.32 mm i.d., d.f. = 0.25 µm) fused silica capillary column connected to a 2 m uncoated piece of fused silica capillary column as "retention gap" was used. The conditions were as follows: temperature, isothermal for 5 min at 60 °C and then from 60 °C to 240 °C at 5 °C/min; carrier gas flow rate, 2.5 ml/min He; temperature of ion source and all connection parts, 200 °C; electron energy, 70 eV; cathodic current, 0.8 mA; injection volumes, 0.5 µl.

Capillary gas chromatography-FTIR spectroscopy (HRGC-FTIR)

Instrument: Nicolet 20 SXB interfaced by a DANI 6500 gas chromatograph equipped with FID. A J & W DB-Wax (30 m × 0.32 mm i.d., d.f. = 0.25 µm) fused silica capillary column was used. Total sample injection mode using PTV (40 °C–240 °C, 0.1 min) was performed. The temperature program was 3 min isothermal at 50 °C and the from 50 °C to 250 °C at 4 °C/min. Light pipe and transfer line were held at 250 °C. He (2.5 ml/min) was employed as carrier gas. Vapour phase FTIR spectra were recorded from 400–4000 cm⁻¹ with a resolution of 8 cm⁻¹. Injection volumes, 0.5 µl.

Reference compounds

(*E,Z*)- (**4**) and (*E,E*)-2,6-dimethylocta-2,6-dien-1,8-diol (**5**): Syntheses were accomplished by SeO₂ oxidation of **2** and **3**, respectively, according to Behr *et al.* [9]. **4**: MS (*m/z*, %): 43 (100), 68 (59), 84 (47), 55 (19), 93 (17), 121 (8), 137 (7), 134 (4). FTIR (vapour phase, *v*, cm⁻¹): 3658, 2931, 2873, 1666, 1450, 1386, 1190, 1008. *R*_t: 2600. **5**: MS (*m/z*, %): 43 (100), 68 (53), 84 (18), 55 (16), 121 (5), 137 (3), 134 (2). FTIR (vapour phase, *v*, cm⁻¹): 3657, 2931, 2873, 1666, 1449, 1386, 1202, 1007. *R*_t: 2648.

All the other reference compounds were available from our own laboratory collection of flavour substances.

Results and Discussion

In grape must (A), synthetic medium (B) as well as mixtures of A and B, citral (**1**) was completely

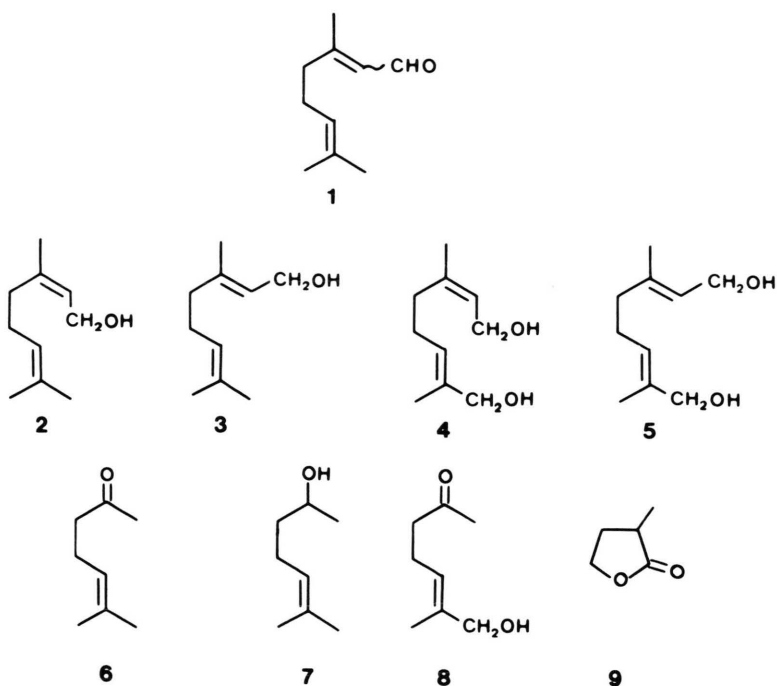


Fig. 1. Structures of bioconversion products formed from citral (**1**) by *Botrytis cinerea* strains. (**2**) nerol; (**3**) geraniol; (**4**) (*E,Z*)-2,6-dimethylocta-2,6-dien-1,8-diol; (**5**) (*E,E*)-2,6-dimethylocta-2,6-dien-1,8-diol; (**6**) 2-methyl-2-hepten-6-one; (**7**) 2-methyl-2-hepten-6-ol; (**8**) 2-methyl-2-hepten-6-one-1-ol; (**9**) 2-methyl-γ-butyrolactone.

metabolized by the four *Botrytis cinerea* strains used. Whereas in A volatile bioconversion products could not be observed, in B and A/B mixtures the compounds **2**–**9** shown in Fig. 1 were identified after extractive sample preparation in the fermentation media by capillary gas chromatography (HRGC), capillary gas chromatography-mass spectrometry (HRGC-MS) and capillary gas chromatography-Fourier transform infrared spectroscopy (HRGC-FTIR). In the acidic fractions of the extracts of fermentation media, biotransformation products of **1** were not detectable. C₁ oxidation has been previous-

ly found in bioconversion studies of **1** using *Pseudomonas convexa* [10]. Geranic acid and other acids have been also detected as biotransformation products from **1** by *Pseudomonas aeruginosa* [11].

As shown from Table I, in B predominant conversion of **1** to nerol (**2**) and geraniol (**3**) occurred; in minor amounts (*E,Z*)- (**4**) and (*E,E*)-2,6-dimethylocta-2,6-dien-1,8-diol (**5**), 2-methyl-2-hepten-6-one (**6**), 2-methyl-2-hepten-6-ol (**7**), 2-methyl-2-hepten-6-one-1-ol (**8**) and 2-methyl-γ-butyrolactone (**9**) were determined. Using small amounts of A in B (1:700 to 5:700) low yields of **2** and **3** were observed, whereas

Table I. Yields (mg/l) and distribution (%) of bioconversion products formed from citral (**1**) by *Botrytis cinerea* strains in a synthetic medium (B) and a 1:700 mixture of grape must/synthetic medium (A/B).

Strain	Medium	Yield [mg/l]	Percentage of yield for compounds							
			2	3	4	5	6	7	8	9
5899/4	B	12.7	32	38	4	10	12	4	tr	tr
	A/B	1.8	4	10	38	32	1	1	10	4
5901/2	B	21.5	31	59	1	1	6	1	< 1	tr
	A/B	5.9	16	1	9	3	56	5	10	1
5882/1	B	29.4	40	54	–	–	6	<1	–	–
	A/B	4.2	18	1	7	2	58	4	10	1
5909/1	B	19.7	30	59	1	2	6	2	tr	–
	A/B	5.3	16	1	14	5	46	5	13	1

tr = traces.

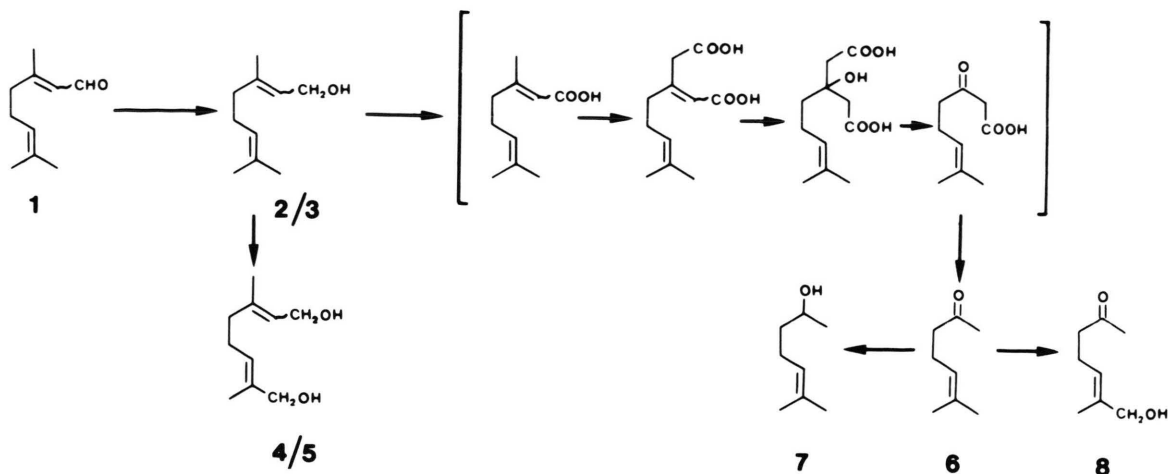


Fig. 2. Potential pathways for the formation of bioconversion products of **1** by *Botrytis cinerea* strains.

the production of **4**, **5**, **6** and **8** increased. Quantitatively, the findings were strongly dependent on the strains used. The results represented in Table I were obtained employing an 1:700 A/B mixture. In order to explain the effect caused by addition of grape must to medium B, a relation between fungal growth — reduced in A/B in comparison to A and quite different in B (**1** as sole carbon source) — and induction of enzymes catalyzing the bioconversion of **1** could be considered. However, this effect was not further studied.

Corresponding to our bioconversion studies with cinnamic aldehyde leading to the formation of cinnamic alcohol by *B. cinerea* [12], in the experiments using **1** also reduction of the aldehyde function was found as predominant metabolic step. Recently, transformation of *E*-2-hexenal to *E*-2-hexenol and 1-hexanol by *B. cinerea* has been also described [13].

From **2** and **3** two main pathways can be discussed to elucidate the formation of other bioconversion products formed from **1** by *B. cinerea*. As schematically shown in Fig. 2, ω -hydroxylation led to **4** and **5**. In plants, ω -hydroxylation of **2** and **3** catalyzed by cytochrome P-450-dependent monooxygenase has been reported [14, 15]. Furthermore, a hypothetical pathway as discussed earlier for bacteria by Seubert and coworkers [16, 17] can be considered leading to **6**, from which reduction led to **7** and ω -hydroxylation to **8**.

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