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

Z. Naturforsch. **42c**, 1097–1100 (1987); received June 3/July 1, 1987

Botrytis cinerea, Citral Biotransformation, Capillary Gas Chromatography, Coupled on-line Capillary Gas Chromatographic Techniques

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, C_1 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].

Reprint requests to Prof. Dr. P. Schreier.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0900–1097 $ 01.30/0

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

a. 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.

b. Synthetic medium. The medium contained (per l): $NaNO_3$ 3 g; K_2HPO_4 1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; KCl 0.5 g and $FeSO_4$ 0.01 g. The pH was adjusted to 3.5 using 1 N HCl.

c. 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

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% $NaHCO_3$ 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 CH_2N_2, 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 N_2 as well as for the detector gases 30 ml/min H_2 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 SeO_2 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, ν, 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, ν, 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

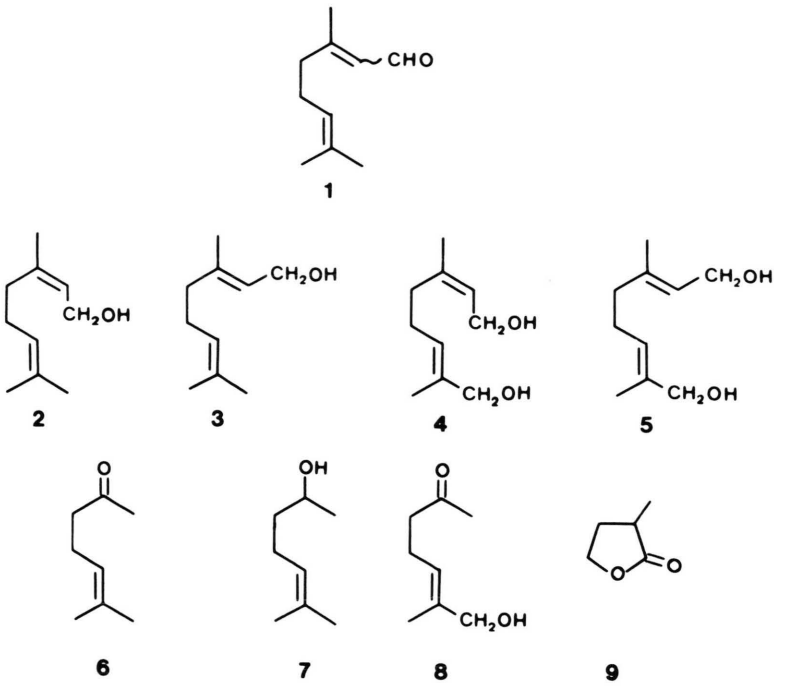

Fig. 1. Structures of bioconversion products formed from citral (**1**) by *Botrytis cinerea* strains. (**2**) nerol; (**3**) geraniol; (**4**) (*E,Z*)-2,6-dimethylocta-2,6-dien-1,8-diol; (**5**) (*E,E*)-2,6-dimethylocta-2,6-dien-1,8-diol; (**6**) 2-methyl-2-hepten-6-on; (**7**) 2-methyl-2-hepten-6-ol; (**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. C_1 oxidation has been previously 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-dimethyl-octa-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.

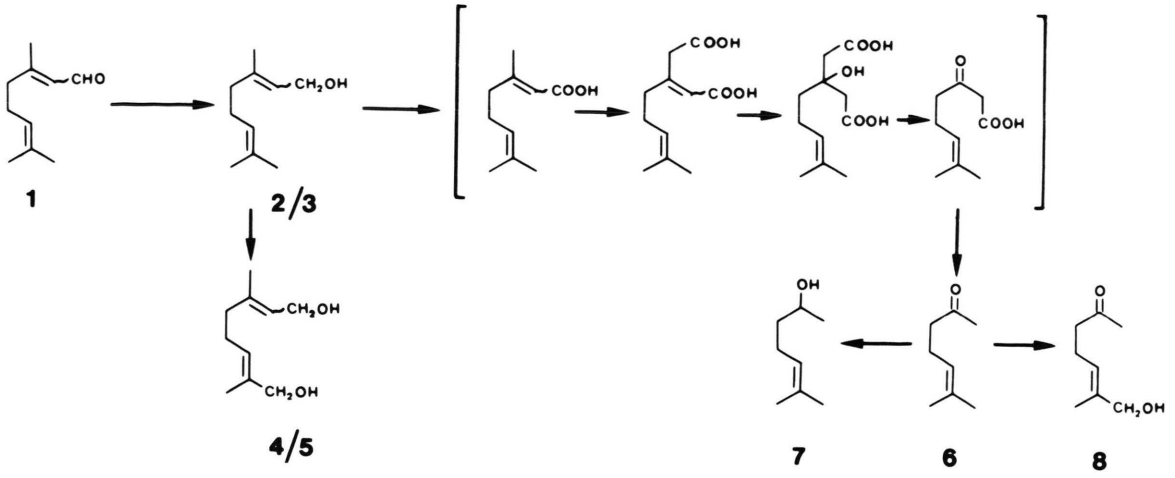

Fig. 2. Potential pathways for the formation of bioconversion products of **1** by *Botrytis cinerea* strains.

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 hypothetic 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**.

Acknowledgements

C. Kahre's skilful operation of HRGC-FTIR analyses is gratefully acknowledged. The authors thank Dr. Lorenz, Neustadt, for providing different *B. cinerea* strains.

[1] J. N. Boidron, Ann. Technol. Agric. **27,** 141 (1978).
[2] J. Shimizu, M. Uehara, and M. Watanabe, Agric. Biol. Chem. **46,** 1339 (1982).
[3] G. Bock, I. Benda, and P. Schreier, J. Food Sci. **51,** 659 (1986).
[4] A. Rapp, H. Mandery, and H. Niebergall, Vitis, Ber. Rebenforsch. **25,** 79 (1986).
[5] P. Brunerie, I. Benda, G. Bock, and P. Schreier, Applied Microbiol. Biotechnol., in press.
[6] R. M. Ikeda, L. A. Rolle, S. H. Vannier, and W. L. Stanley, J. Agric. Food Chem. **10,** 98 (1962).
[7] K. Bauer and D. Garbe, Common fragrance and flavor materials. Preparation, properties and uses, VCH Verlagsgesellschaft, Weinheim 1985.
[8] F. Drawert and A. Rapp, Chromatographia **1,** 446 (1968).
[9] D. Behr, I. Wahlberg, T. Nishida, and C. R. Enzell, Acta Chem. Scand. **32B,** 228 (1978).
[10] T. Hayashi, H. Takashiba, H. Ueda, and C. Tatsumi, J. Agric. Chem. Soc. Japan **41,** 254 (1967).
[11] S. S. Joglekar and R. S. Dhavlikar, Appl. Microbiol. **18,** 1084 (1969).
[12] G. Bock, I. Benda, and P. Schreier, Z. Lebensm. Unters.-Forsch., in press.
[13] I. Urbasch, Z. Naturforsch. **42c,** 64 (1987).
[14] T. D. Meehan and C. J. Coscia, Biochem. Biophys. Res. Commun. **53,** 1043 (1973).
[15] H. Fretz and W. D. Woggon, Helv. Chim. Acta **69,** 1959 (1986).
[16] W. Seubert and U. Remberger, Biochem. Z. **338,** 245 (1963).
[17] W. Seubert and E. Fass, Biochem. Z. **341,** 23 (1964).