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Bioconversion of α-Damascone by *Botrytis cinerea*

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Bioconversion of α-damascone (compound 1) was studied with four strains of *Botrytis cinerea* in grape must (pH 3.2). As biotransformation products of compound 1, 3-oxo-α-damascone, *cis*- and *trans*-3-hydroxy-α-damascone, γ-damascenone, 3-oxo-8, 9-dihydro-α-damascone, and *cis*- and *trans*-3-hydroxy-8,9-dihydro-α-damascone were identified. In addition, acid-catalyzed chemical transformation of compound 1 to the diastereomers of 9-hydroxy-8,9-dihydro-α-damascone was observed. Identifications were performed by capillary gas chromatography (HRGC) and coupled HRGC techniques, i.e., on-line HRGC-mass spectrometry and HRGC-Fourier transform infrared spectroscopy, after extractive sample preparation.

C_{13}-cyclic terpenoid ketones such as ionones and damascones are natural aroma components. For example, α-ionone has been identified as a flavor ingredient of raspberry, carrot, and black tea as well as in numerous plant tissues. α-Damascone (compound 1) has been described as a flavor constituent of black tea and tobacco (11). The C_{13} norisoprenoids are regarded to be generated from carotenoids by complex enzymic and nonenzymic reactions (5).

The damascones exhibit fruity, roselike odors that are desired in various flavor and fragrance compositions (1). Because of their attractive sensory properties, there has been considerable industrial interest in C_{13} norisoprenoids and their derivatives (10). Recently, the usefulness of microbial conversions for the preparation of this class of compounds has been demonstrated with several ionones as starting material (17). This paper concerns the microbial bioconversion of compound 1 by *Botrytis cinerea*. In this study, we dealt mainly with the identification of conversion products. In our previous work, *B. cinerea* was found to be a useful microorganism for the biotransformation of monoterpene alcohols (2–4).

MATERIALS AND METHODS

Chemicals. All commercial chemicals used were of analytical grade. Solvents were redistilled before use. Compound 1 was a donated sample from Dragoco GmbH, Holzminden, Federal Republic of Germany.

B. cinerea strains. *B. cinerea* 5882/1, 5899/4, 5901/2, and 5909/1 were obtained from the collection of the Bayerische Landesanstalt für Weinbau und Gartenbau, Würzburg, Federal Republic of Germany. From the original cultures, portions were transferred to malt agar slants and incubated at 25°C for 7 days.

Medium and incubation conditions. Grape must (cv. Müller-Thurgau) with sugar and acid contents of 193 and 8.0 g/liter (pH 3.2), respectively, was used. Grape must (700 ml) was placed in 1-liter Erlenmeyer flasks and sterilized (30 min at 110°C). After the addition of 50 mg of compound 1 per liter (in 1 ml of ethanol), each flask was inoculated with a pure *B. cinerea* strain and incubated at 25°C for 14 days. The mycelium was removed by filtration, and the solutions were 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 both without *B. cinerea* and without compound 1 were carried out.

In additional experiments, the time course of the bioconversion of compound 1 was studied by variation of incubation times from 1 to 14 days.

Isolation of bioconversion products. After the addition of an internal standard (2-methyl-1-pentanol, 0.4 mg/l) to the above-mentioned filtrates, solvent extraction was carried out continuously over 24 h with pentane-dichloromethane (2:1). The organic phase was dried over Na_2SO_4 sicc. and carefully concentrated to 1 ml with a Vigreux column (45°C) for subsequent HRGC, HRGC-MS, and HRGC-FTIR analyses.

HRGC. A Carlo Erba Fractovap 4100 gas chromatograph equipped with a flame ionization detector and a J & W fused silica DB-Wax capillary column (30 m by 0.259 mm [inner diameter]; df, 0.25 μm) was used. Split injection (1:20) was used. The temperature program was isothermal for 3 min at 50°C and then 50 to 240°C at 4°C/min. The flow rates were 2.0 ml/min for the carrier gas He, 30 ml/min for the make-up gas N_2, and 30 and 300 ml/min for the detector gases H_2 and air, respectively. The injector and detector temperatures were both kept at 300°C.

Results of qualitative analyses were verified by comparison of the HRGC retention (R_i), mass spectra, and, in part, vapor-phase FTIR spectra with those of synthesized reference compounds. Quantitative HRGC determinations were carried out by standard controlled calculations with a Shimadzu CR-6-A computing integrator without consideration of calibration factors (F, 1.00).

HRGC-MS. A Varian aerograph 1440 gas chromatograph directly coupled to a Finnigan MAT 44 mass spectrometer with a PCDS data system was used. The apparatus was equipped with a J & W fused-silica DB-Wax capillary column (30 m by 0.259 mm [inner diameter]; df, 0.25 μm). Split injection (1:10) was used. The conditions were as follows: temperature program, 50 to 240°C at 4°C/min; flow rate for the carrier gas He, 2.5 ml/min; temperature of the ion source and all connection parts, 200°C; electron energy, 70 eV; cathodic current, 0.8 mA.

HRGC-FTIR. A Nicolet 20 SXB system interfaced with a Dani 6500 gas chromatograph equipped with a flame ionization detector was used. A J & W DB-Wax fused silica capillary column (30 m by 0.32 mm [inner diameter]; df, 0.25

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μm) was used. The total sample injection mode with PTV (40 to 240°C, 0.1 min) was used. The temperature program was 50 to 240°C at 4°C/min. The light pipe and transfer line were kept at 240°C. He (2.5 ml/min) was used as the carrier gas. Vapor-phase FTIR spectra were recorded from 400 to 4,000 cm^{-1} with a resolution of 8 cm^{-1}.

Preparative HPLC. Preparative high-performance liquid chromatography (HPLC) was performed with a silica gel column (250 by 16 mm) packed with 5-μm particles (Knauer). A model 64 HPLC pump (Knauer) and a 230-nm UV detector (Knauer) were used. Eluent mixtures contained hexane and *tert*-butyl methyl ether (details are given below for chemical syntheses). The flow rates were 10 ml/min.

NMR spectra. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AC 200 (200-MHz) and WM 400 (400-MHz) spectrometers with $CDCl_3$ and benzene-d_6 as solvents and Me_4Si as an internal standard.

Reference compounds. (i) 9-Hydroxy-8,9-dihydro-α-damascone (compound 2A/2B) (mixture of diastereomers). Five grams of compound 1 in 200 ml of H_2O (pH 1.0) was stirred for 2 weeks at room temperature. After solvent extraction of the liquid with pentane-dichloromethane (2:1), LC purification on silica gel with pentane-diethyl ether (5:1) and, after elution of compound 1, pentane-diethyl ether (3:1) as eluents was performed. R_i 2099 (2A), 2105 (2B). MS (nearly identical for 2A and 2B) (*m/z*, percent) 43 (100), 45 (49), 87 (48), 81 (23), 123 (20), 109 (10), 67 (10), 69 (8), 55 (8), 210 (6). FTIR (vapor phase, ν, cm^{-1}) 3592, 3031, 2969, 2927, 2886, 1710, 1631 (w). ^1H NMR (AC 200, $CDCl_3$ ppm) 0.92 (3H, s), 0.93 (3H, s), 1.22 (3H, m), 1.60 (3H, s), 2.48 (1H, m), 2.73 (1H, br, s), 2.79 (1H, m), 4.20 (1H, m), 5.61 (1H, br, s). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 22.1, 22.5, 23.3, 27.7, 27.9, 29.3, 30.6, 33.6, 53.1, 63.8, 123.7, 129.6, 215.8 (for 30.6, 63.8, 123.7, 129.6, and 215.8, the signal was split, indicating the occurrence of diastereomers).

(ii) 3-Oxo-α-damascone (compound 3). Synthesis of compound 3 was accomplished by catalytic oxidation of compound 1 as described previously (7). The purification was performed by LC on silica gel with pentane-diethyl ether (4:1) as the eluent. R_i 2439. MS (*m/z*, percent) 69 (100), 41 (50), 123 (15), 138 (13), 79 (8), 77 (8), 206 (5). FTIR (vapor phase, ν, cm^{-1}) 3035, 2970, 2925, 2886, 1694, 1633, 966. ^1H NMR (AC 200, $CDCl_3$, ppm) 1.00 (3H, s), 1.10 (3H, s), 1.86 (3H, d, J = 1.3 Hz), 1.96 (3H, dxd, J_1 = 6.8 Hz, J_2 = 1.6 Hz), 2.05 (1H, d, J = 16.6 Hz), 2.68 (1H, d, J = 16.6 Hz), 3.39 (1H, br, s), 6.01 (1H, br, s), 6.29 (1H, dxq, J_1 = 15.5 Hz, J_2 = 1.7 Hz), 6.99 (1H, dxq, J_1 = 15.5 Hz, J_2 = 6.8 Hz). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 18.3, 23.8, 27.5, 29.1, 36.6, 47.0, 61.7, 127.5, 132.4, 144.8, 156.1, 197.7, 199.1.

(iii) *cis*- and *trans*-3-Hydroxy-α-damascone (compounds 4 and 5, respectively). Compounds 4 and 5 were prepared by bromination of compound 1 with *N*-bromosuccinimide as described previously (9) and substitution of Br^- with OH^- by stirring the bromo compounds in a 10% Na_2CO_3 solution for 2 days as described previously (6). To isolate the two compounds, we performed LC purification on silica gel with pentane-diethyl ether (5:1) and, after elution of compound 1, pentane-diethyl ether (1:1). Finally, preparative HPLC with a silica gel column and hexane–tert-butyl methyl ether (1:1) as the eluent was used to separate the two diastereomers. Compound 4. R_i 2412. MS (*m/z*, percent) 69 (100), 107 (95), 41 (75), 122 (40), 43 (30), 91 (18), 55 (10), 151 (10), 208 (5). FTIR (vapor phase, ν, cm^{-1}) 3642, 3611, 3085, 3038, 2968, 2929, 2879, 1694, 1633, 1169, 1029, 966. ^1H NMR (WM 400, benzene-d_6, ppm) 0.79 (3H, s), 0.83 (3H, s), 1.33 (3H, d, J = 6.8 Hz), 1.48 (3H, s), 1.54 (1H, dxd, J_1 = 12.8 Hz, J_2 = 6.5 Hz), 1.88 (1H, dxd, J_1 = 12.8 Hz, J_2 = 9.8 Hz), 2.75 (1H, br, s), 4.13 (1H, m), 5.67 (1H, br, s), 6. 15 (1H, dxq, J_1 = 15.5 Hz, J_2 = 1.7 Hz), 6.68 (1H, dxq, J_1 = 15.5 Hz, J_2 = 6.8 Hz). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 18.3, 23.0, 28.3, 28.6, 34.9, 40.9, 60.8, 66.1, 127.7, 132.4, 133.3, 143.3, 201.4. Compound 5. R_i 2463. MS (*m/z*, percent) 69 (100), 41 (70), 107 (38), 43 (25), 109 (20), 151 (18), 123 (16), 122 (15), 208 (5). FTIR (vapor phase, ν, cm^{-1}) 3650, 3080, 3036, 2965, 2925, 2885, 1693, 1632, 1158, 1033, 966. ^1H NMR (WM 400, $CDCl_3$, ppm) 0.93 (3H, s), 1.12 (3H, s), 1.40 (1H, dxd, J_1 = 13.6 Hz, J_2 = 5.4 Hz), 1.63 (3H, s), 1.88 (3H, d, J = 6.8 Hz), 1.94 (1H, dxd, J_1 = 13.6 Hz, J_2 = 6.0 Hz), 3.13 (1H, br, s), 4.35 (1H, m), 5.71 (1H, br, s), 6.23 (1H, dxq, J_1 = 15.5 Hz, J_2 = 1.7 Hz), 6.90 (1H, dxq, J_1 = 15.5 Hz, J_2 = 6.8 Hz). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 18.2, 22.6, 25.8, 30.5, 33.3, 43.6, 61.0, 65.4, 126.7, 132.6, 134.0, 142.7, 200.4.

(iv) γ-Damascenone (compound 6). Compound 6 was synthesized by bromination of compound 1 with *N*-bromosuccinimide and dehydrobromination of the resulting bromo compounds with diethylaniline as previously reported (8). LC purification on a silica gel with pentane-diethyl ether (5:1) as the eluent and preparative HPLC separation on silica gel with hexane–*tert*-butyl methyl ether (97.5:2.5) as the eluent were used to isolate compound 6. R_i 1830. MS (*m/z*, percent) 69 (100), 41 (50), 91 (18), 121 (18), 77 (16), 105 (16), 55 (10), 175 (10), 190 (8). FTIR (vapor phase, ν, cm^{-1}) 3086, 3038, 2962, 2929, 2905, 2843, 1692, 1635, 1601, 1319, 1290, 1070, 965, 887. ^1H NMR (AC 200, $CDCl_3$, ppm) 0.92 (3H, s), 0.97 (3H, s), 1.88 (3H, dxd, J_1 = 6.8 Hz; J_2 = 1.6 Hz), 2.40 (1H, d, J = 18.3 Hz), (signal of the corresponding diastereotope proton under dxd, 1.88 ppm), 3.24 (1H, s), 4.82 (1H, br, s), 5.02 (1H, br, s), 5.83 (1H, m), 6.15 (1H, m), 6.22 (1H, dxq, J_1 = 15.4 Hz, J_2 = 1.7 Hz), 6.88 (1H, dxq, J_1 = 15.4 Hz, J_2 = 6.8 Hz). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 18.1, 27.3, 28.8, 32.4, 37.1, 61.4, 115.3, 127.0, 129.5, 131.4, 140.4, 142.5, 199.3.

(v) 3-Oxo-8,9-dihydro-α-damascone (compound 7). One hundred milligrams of compound 3 dissolved in 20 ml of ethanol (absolute) containing 200 mg of Pd on $BaSO_4$ as a catalyst was stirred in an H_2 atmosphere for 10 min. After filtration, preparative HPLC separation on silica gel with hexane–*tert*-butyl methyl ether (3:2) was used to separate compound 7 from the remaining educt compound 3. R_i 2300. MS (*m/z*, percent) 123 (100), 138 (40), 43 (30), 71 (28), 41 (10). FTIR (vapor phase, ν, cm^{-1}) 3035 (w), 2971, 2889, 1696, 1638 (w). ^1H NMR (AC 200, $CDCl_3$, ppm) 0.95 (3H, t, J = 7.5 Hz), 1.05 (3H, s), 1.08 (3H, s), 1.63 (2H, m), 1.88 (3H, d, J = 1.3 Hz), 2.02 (1H, d, J = 16.7 Hz), 2.62 (1H, d, J = 16.7 Hz), 2.62 (2H, m), 3.21 (1H, br, s), 5.97 (1H, br, s). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 13.5, 16.6, 23.9, 27.4, 28.9, 36.7, 46.5, 48.8, 64.2, 127.2, 155.3, 198.9, 208.6.

(vi) *cis*- and *trans*-3-Hydroxy-8,9-dihydro-α-damascone (compounds 8 and 9, respectively). Direct hydrogenation of compounds 4 and 5 by synthesis procedure used for compound 7 failed. Destruction of the compounds during the elimination of water was observed. Thus, 8,9-dihydro-α-damascone was synthesized by hydrogenation of compound 1 as described above for the synthesis of compound 7 and purified by LC on silica gel with pentane-diethyl ether (9:1) as the eluent. Bromination of 8,9-dihydro-α-damascone and substitution of Br^- with OH^- (as in the syntheses of compounds 4 and 5) yielded compounds 8 and 9. LC purification on silica gel with pentane-diethyl ether (5:1) and, after elution of the remaining 8,9-dihydro-α-damascone, with pentane-diethyl ether (2:1) as well as preparative HPLC separation on silica gel with hexane–*tert*-butyl methyl ether

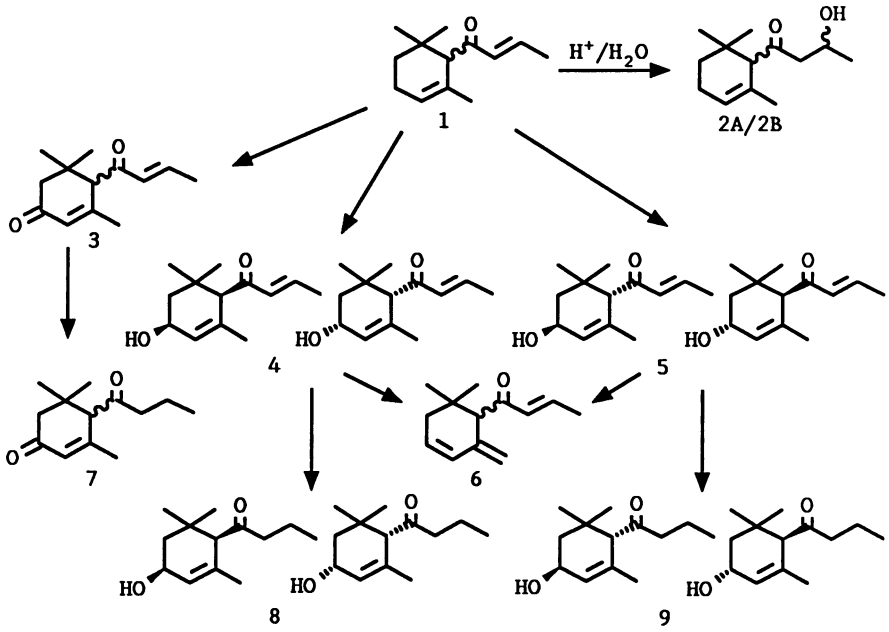

FIG. 1. Bioconversion products formed from compound 1 by *B. cinerea* and their potential formation pathways. The spectroscopic differentiation of *cis* and *trans* isomeric compounds 4 and 5 has been described elsewhere (13).

(3:2) supplied pure compounds 8 and 9. Compound 8. R_i 2281. MS (*m/z*, percent) 107 (100), 43 (98), 41 (50), 71 (35), 122 (35), 55 (17), 91 (17), 69 (10), 192 (5). FTIR (vapor phase, ν, cm^{-1}) 3635, 3037, 2969, 1715, 1652, 1367, 1027. ^1H NMR (AC 200, $CDCl_3$, ppm) 0.92 (3H, t, J = 7.0 Hz), 0.92 (3H, s), 0.96 (3H, s), 1.60 (3H, s), 1.60 (2H, d, J = 3.4 Hz), 1.62 (2H, m), 2.50 (2H, m), 2.77 (1H, br, s), 4.18 (1H, m), 5.67 (1H, br, s). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 13.6, 16.6, 23.0, 28.1, 28.3, 34.9, 40.3, 47.9, 63.1, 65.8, 127.7, 132.6, 212.8. Compound 9. R_i 2313. MS (*m/z*, percent) 43 (100), 41 (50), 107 (40), 71 (37), 83 (22), 121 (18), 122 (15). FTIR (vapor phase, ν, cm^{-1}) 3642, 3038, 2969, 1716, 1643, 1365, 1026. ^1H NMR (AC 200, $CDCl_3$, ppm) 0.90 (3H, s), 0.92 (3H, t, J = 7.3 Hz), 1.12 (3H, s), 1.40 (1H, dxd, J_1 = 13.9 Hz, J_2 = 4.5 Hz), 1.60 (2H, m) 1.61 (3H, s), 1.95 (1H, dxd, J_1 = 13.9 Hz, J_2 = 6.0 Hz), 2.48 (2H, m), 2.99 (1H, br, s), 4.31 (1H, m), 5.67 (1H, br, s). ^{13}C NMR (AC 200, $CDCl_3$, ppm) 13.6, 16.7, 22.8, 26.2, 30.3, 32.9, 42.4, 48.4, 63.0, 65.0, 126.1, 133.6, 211.9.

RESULTS AND DISCUSSION

All four *B. cinerea* strains under study were able to transform compound 1. With grape must (pH 3.2), compound 1 was completely metabolized by the fungus. HRGC, HRGC-MS, and HRGC-FTIR analyses revealed the occurrence of the biotransformation compounds 3, compounds 4 and 5, compound 6, compound 7, and compounds 8 and 9 (Fig. 1). In blank tests carried out without *B. cinerea* inoculation, acid-catalyzed chemical conversion of compound 1 to the diastereomers 2A and 2B was observed.

Different yields and distributions of bioconversion products were obtained, depending on the *B. cinerea* strains used (Table 1). Whereas two strains, 5899/4 and 5909/1, predominantly produced the hydrogenated compounds 7, 8, and 9, the two remaining strains, 5882/1 and 5901/2, predominantly produced compounds 3, 4, and 5. Strain 5901/2 mainly transformed compound 1 to compound 6.

Time course studies of the metabolization of compound 1 by *B. cinerea* 5901/2 revealed a maximal formation of bioconversion products after 9 days of incubation. Since the 3-oxygenated compounds 3, 4, and 5 were the first detectable derivatives of compound 1, they can be regarded as its primary conversion products. The hydrogenated compounds 7, 8, and 9 appeared after 8 days of incubation, with a maximal formation at day 13 (Fig. 2). Because of these observations, we postulated the pathways for the microbial transformation products of compound 1 as schematically outlined in Fig. 1.

Microbial conversion of compound 1 has not been carried out as yet. Recently, Yamazaki et al. (17) studied the biotransformation of its isomer, i.e., α-ionone, by *Aspergillus niger* and were able to identify the derivatives 3-oxo-α-ionone, *cis*- and *trans*-3-hydroxy-α-ionone, and 1-(6, 6-dimethyl-2-methylene-3-cyclohexyl)-buten-3-one, which correspond to compounds 3, 4, 5, and 6, respectively, found in our study.

TABLE 1. Yields of bioconversion products 3 to 9 and chemically formed products 2A and 2B as well as the distribution of products 3 to 9 obtained from compound 1 (50 mg/liter) by *B. cinerea* strains in grape must[a]

Strain	Yield (mg/liter)[b] of products:		Distribution (%) of products:						
	3 to 9	2A and 2B	3	4	5	6	7	8	9
5899/4	1.1	0.2	8	8	12	11	33	22	6
5901/2	0.5	0.4	8	19	16	32	11	10	3
5882/1	0.5	0.9	13	26	42	6	6	7	
5909/1	0.6	0.6	7	9	13	3	21	29	18

[a] Incubation was done for 14 days. The data presented are mean values of two incubation experiments (variation, ±12%).

[b] Standard calculated values without consideration of extraction yields (*F*, 1.0).

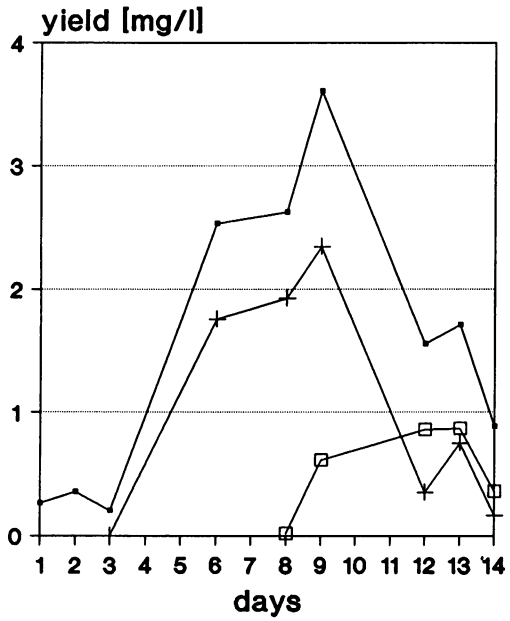

FIG. 2. Yields of products obtained during time course studies of the bioconversion of compound 1 by *B. cinerea*. Symbols: ■, total products; +, products 3, 4, and 5; □, products 7, 8, and 9.

However, hydrogenation in the side chain of the educt has not been observed.

Among the bioconversion products identified in our study, compounds 7, 8, and 9 have not been described yet, whereas compounds 2, 3, 4, 5, and 6 are known flavor substances (12, 14–16).

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