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# Optimization of a biocatalytic single-step alkene cleavage of aryl alkenes

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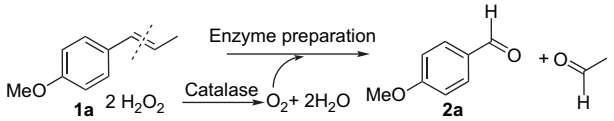
**Abstract**—The oxidative cleavage of a C=C double bond adjacent to an aryl moiety was achieved in the presence of a protein preparation of *Trametes hirsuta* G FCC 047 to yield the corresponding aldehydes. Molecular oxygen was the only oxidant required. All positive substrates had a C=C bond conjugated to an aromatic system, all other compounds tested not fulfilling this requirement were non-substrates. The optimum reaction conditions are 20 °C, pH 6–6.5, 15% v/v ethanol as co-solvent at an apparent oxygen pressure of 2 bar.


## 1. Introduction

Alkene cleavage leading to aldehydes or ketones is a very frequently used method in synthetic organic chemistry.[1–3] Among the methods currently available for the chemical oxidative cleavage of alkenes, reductive ozonolysis is the most frequently employed. Biotechnological methods at the moment have a rather low significance.[4] Other methods involving metal-based oxidants like the Lemieux–Johnson reagent[5] require (at least) stoichiometric amounts of salts or peroxides, and are plagued by limited chemo-, regio-, and stereoselectivities. Over-oxidation of the aldehyde to the corresponding acid as side-reaction is in most cases unavoidable. The only catalytic method employing molecular oxygen needs Co(II) as catalyst and is restricted to isoeugenol-type substrates displaying moderate chemo-selectivity.[6] Despite mild and selective oxidation methods gaining increased importance,[7–10] only few novel or improved methods for alkene cleavage are available.[11–15] Very recently, we reported on a highly selective and mild biocatalytic method for the cleavage of aryl alkenes employing molecular oxygen as the oxidant to give the corresponding aldehydes or ketones.[16] In this paper we describe the full details on the optimization of the sustainable transformation, where molecular oxygen is activated and consumed.

## 2. Results and discussion

*trans*-Anethole **1a** was employed as a model substrate, which was cleaved to give anisaldehyde **2a** (Scheme 1). To reach an overpressure of molecular oxygen on a milliliter scale, the disproportion of hydrogen peroxide to yield molecular oxygen and water catalyzed by a catalase was exploited. Furthermore, to ensure preparative significance for a biocatalytic transformation, the substrate concentration was set to 6 g/l.




**Scheme 1**. Biocatalytic alkene cleavage of *trans*-anethole with molecular oxygen.

### 2.1. Temperature

Cell free extract of *Trametes hirsuta* G FCC 047 was used for the optimization of reaction parameters, to avoid the interference of mass transport effects through the cell wall. As

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can be seen from Figure 1, temperatures between 15 and 30 °C are appropriate showing a maximum at 20 °C.

### 2.2. pH

Subsequently, the possible pH range was found to be rather narrow (Fig. 2), best activity was found for a pH between 6.0 and 6.5. At pH 7 the catalyst showed 40% residual activity compared to pH 6.5. More basic or acidic conditions led to enzyme deactivation.

### 2.3. Co-solvent

To test the influence of possible water-miscible co-solvents, which are used to increase the solubility of the substrate in the aqueous phase, the reaction was performed with 1.7% v/v of co-solvent employing cell free extract. By plotting the activity against log *P* of the solvent (for the blank, thus without co-solvent the activity was 0.45 mmol/h g protein) no clear general trend (Fig. 3) was observed. The use of ethanol or DMSO resulted in an increase of activity. Other solvents like DMF, acetone, dioxane or 2-propanol caused lower activity. Methanol and 2-propanol proved to be inappropriate.

### 2.4. Ethanol and substrate concentration

Since ethanol was the best co-solvent, it was used for further optimization of the reaction conditions. Variation of the ethanol concentration showed an optimum at 15% v/v (Fig. 4). Keeping the concentration of the co-solvent ethanol at 15% v/v, the substrate concentration was varied and can be increased at least up to apparent 2.7 mol/l (400 g/l) (Fig. 5). The high applicable substrate concentration stresses the potential of the method for preparative transformations.

### 2.5. Hydrogen peroxide

Since the catalase present in the enzyme preparation disproportionates the hydrogen peroxide to water and oxygen within seconds, the amount of hydrogen peroxide added determines the final 'pressure' of molecular oxygen. Therefore

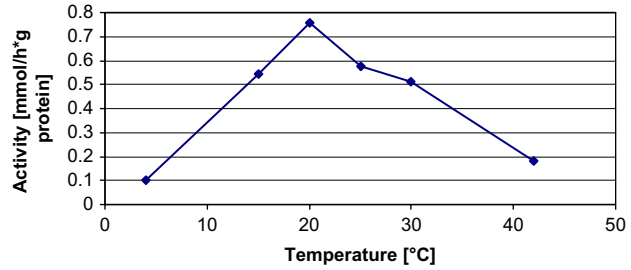



**Figure 1**. Optimum temperature of the alkene cleavage reaction.

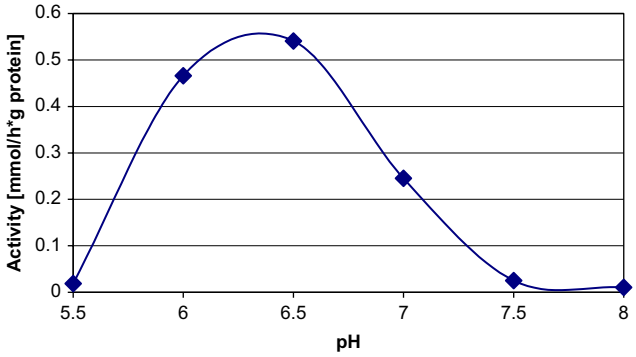



**Figure 2**. Activity profile at varied pH.

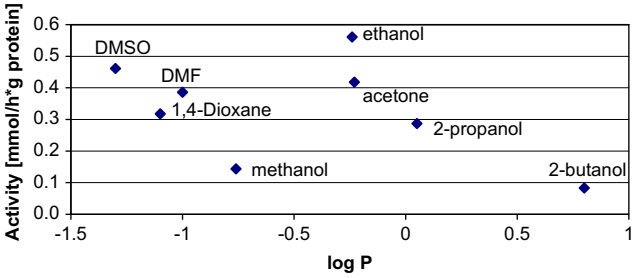



**Figure 3**. Activity in the presence of various co-solvents (1.7% v/v). Without co-solvent the activity was 0.45 mmol/h g protein.

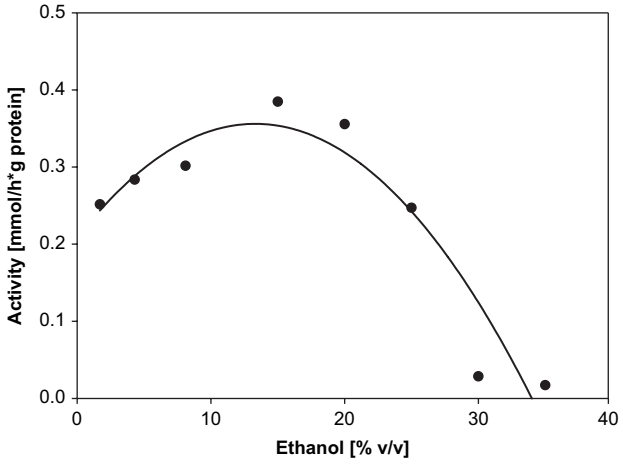



**Figure 4**. Activity of the cell free protein preparation for the alkene cleavage of *trans*-anethole **1a** at varied concentration of ethanol as co-solvent at 40 mM substrate concentration.

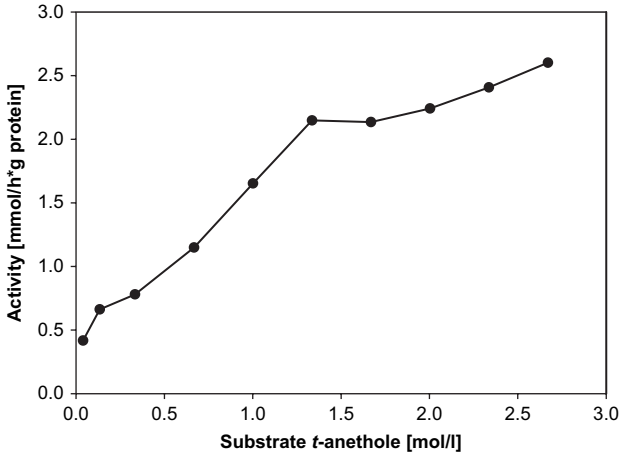



**Figure 5**. Activity of the cell free protein preparation for the alkene cleavage of *trans*-anethole **1a** at varied substrate concentration at 15% v/v ethanol as co-solvent.

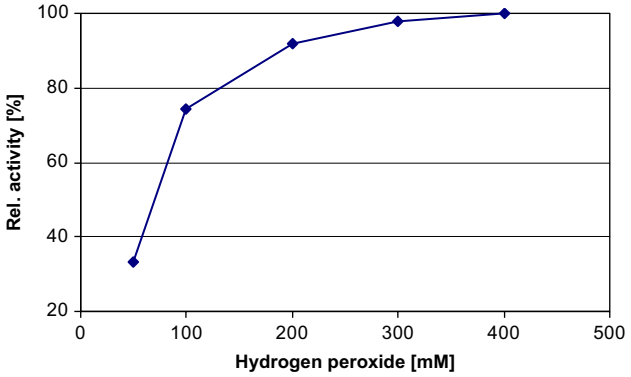



**Figure 6**. Conversion of *trans*-anethole to *p*-anisaldehyde using varied concentration of hydrogen peroxide solution.

varying the initial concentration of hydrogen peroxide by adding the same amount of hydrogen peroxide solutions with different concentrations (initial concentration in the reaction mixture between 50 mM and 400 mM), the activities were compared. The activity increased steeply up to 200 mM initial hydrogen peroxide concentration and seemed to approach a plateau at higher concentrations (Fig. 6). To make sure, that when adding a high concentration of hydrogen peroxide no enzyme is deactivated, the experiment was repeated by adding various amounts of 1 M hydrogen peroxide solution. The same results were obtained as in the previous experiment, indicating that no enzyme deactivation occurred during addition.

## 2.6. Tubes

As a last step of optimization, experiments were carried out in GC-vials (1.5 ml), which were closed with a rubber seal, since it was noticed that the oxygen pressure decreased more rapidly in the Eppendorf tubes than in sealed glass GC-vials. Indeed, by performing the experiments in closed glass GC-tubes the conversion of *trans*-anethole was increased from 30% to 83%. The oxygen pressure could not be determined due to technical reasons, but according to calculations the pressure is approximately 2 bar. The amount of oxygen obtained from 100 µl of a 1 M solution of $H_2O_2$ leads formerly to an oxygen excess of approximately 20% based on substrate concentration (~6 g/l). This indicates that oxygen may get rather quick limiting; therefore the design of improved reactors to control the oxygen pressure is in progress in our laboratory.

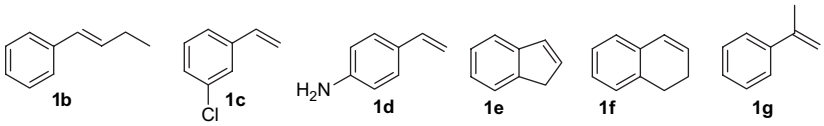
## 2.7. Substrates and non-substrates

Several substrates like *n*-alkenes (e.g., 1-decene and 4-bromo-1-butene), cyclic alkenes (cyclooctene), substrates with isolated double bonds (e.g., 3-chloro-2-methyl-1-propene, limonene, and allyl benzene) or substrates with conjugated double bounds (e.g., myrcene) were tested. Due to the high specificity of the enzyme all of them were non-substrates. Only in the case where an aromatic ring system is in conjugation with a double bond, conversion was observed under the reaction conditions employed. A selection of accepted substrates is given in Table 1. Due to the mild reaction conditions, the catalyst showed excellent chemoselectivity up to >99%, which is rather encouraging for such an oxidation reaction. For instance, *p*-amino styrene **1d** bearing an amino group was selectively cleaved (>99%) to the corresponding aldehyde. In separate experiments it was tested, whether other functional groups susceptible to oxidation like sulfur or nitrogen could be oxidized. However, neither thioanisole, aniline nor *N*-methyl aniline were oxidized, which emphasized the specificity of the method. Indene **1e** bearing a C=C double bond in a five-membered ring was a very good substrate, as was 1,2-dihydronaphthalene **1f**. Nevertheless, substrates fitting this pattern but bearing a polar moiety like cinnamic acid or ferulic acid were also not accepted. Furthermore, *trans*-stilbene was also a non-substrate. Finally, 1,1-disubstituted alkene like α-methyl styrene **1g** was cleaved to the corresponding ketone, acetophenone, which demonstrates that ketones as well as aldehydes are accessible.

## 2.8. Preparative scale

Finally, for demonstrating the biocatalytic alkene cleavage on a preparative scale 590 mg of *trans*-anethole **1a** were transformed under constant controlled oxygen pressure of 2 bar. The substrate **1a** was cleaved with 81% conversion within 24 h. Column chromatography gave 0.31 g (57%) of isolated pure *p*-anisaldehyde.

**Table 1**. GC-yield of obtained aldehydes/ketones after 24 h reaction time




| Substrate | Product | GC-yield (%) | Chemo-selectivity (%) |
|---|---|---|---|
| *trans*-Anethole **1a** | *p*-Anisaldehyde **2a** | 83 | 94 |
| (*E*)-1-Phenyl-1-butene **1b** | Benzaldehyde | 44 | 72[a] |
| 3-Chloro styrene **1c** | 3-Chloro benzaldehyde | 20 | 88 |
| Styrene | Benzaldehyde | 25 | 94 |
| *p*-Amino styrene **1d** | *p*-Amino benzaldehyde | 11 | >99 |
| Indene **1e** | 2-(2-Oxo-ethyl)-benzaldehyde | 71 | 88[b] |
| 1,2-Dihydronaphthalene **1f** | 2-(3-Oxo-propyl)-benzaldehyde | 27 | 63 |
| α-Methyl styrene **1g** | Acetophenone | 26 | >99 |

The chemo-selectivity is given as the ratio of formed aldehyde/ketone to all novel formed compounds.
[a] Identified side-products were 2-ethyl-3-phenyl oxirane (4%), 4-phenyl-3-butene-2-one (17%), and 4-phenyl-3-butene-2-ol (3%), unidentified product (4%).
[b] Identified side-products were 1,2-dihydroxy indane (10%), unidentified product (2%).

## 3. Experimental

### 3.1. General

NMR spectra were recorded in $CDCl_3$ using a Bruker AMX 360 at 360 ($^1$H) and 90 ($^{13}$C) MHz. Chemical shifts are reported relative to TMS ($\delta$ 0.00), coupling constants ($J$) are given in hertz. TLC plates were run on silica gel Merck 60 (F254) and compounds were visualized by standard techniques. Aldehydes were visualized using 2,4-dinitrophenylhydrazine (0.4% w/v in 2 N HCl). Flash chromatography was performed on silica gel Merck 60 (230–400 mesh). Petroleum ether, acetone, and ethyl acetate were used as an eluent. Solvents were dried and freshly distilled by common practice. Petroleum ether (p.e.) had a boiling range of 60–90 °C.

Cells of *T. hirsuta* G FCC 047 were prepared as described previously.[16] The biocatalytic alkene cleavage could be performed with either freshly harvested cells or lyophilized cells. However, lyophilization of a cell free preparation led to complete loss of activity.

**3.1.1. General procedure for biocatalytic alkene cleavage.** Lyophilized cells (25 mg) of *T. hirsuta* G FCC 047 were rehydrated in Bis-Tris buffer (900 µl, 50 mM, pH 6) for 30 min on a rotary shaker (150 rpm). Cells were removed by centrifugation (13,000 rpm, 2 min) and supernatant (900 µl, 1 mg protein/ml) was transferred into an Eppendorf vial (1.5 ml). The biotransformation of *trans*-anethole (6 µl, 5.9 mg, 40 µmol) was started by the addition of $H_2O_2$ (100 µl, 1 M) and quick closing of the vial. The mixture was shaken on a rotary shaker (150 rpm) at 25 °C. After 24 h the mixture was extracted with EtOAc (2×500 µl) containing toluene as an internal standard (6 µl toluene/ml EtOAc) and centrifuged (13,000 rpm, 2 min) for phase separation. The combined organic layers were dried over $Na_2SO_4$ and analyzed by GC and GC–MS. To test for possible acid formation the solution was acidified prior to work up in separate experiments.

**3.1.1.1. Optimization of temperature.** Experiments were performed as described in the general procedure but the reactions were incubated at different temperatures (4, 15, 20, 25, 30, 42 °C). For measuring activity, thus initial rates, the experiments were stopped at conversion below 20% to be in the linear range of kinetics (4–6 h).

**3.1.1.2. Optimization of pH.** Lyophilized cells (25–30 mg) of *T. hirsuta* G FCC 047 were rehydrated in the appropriate buffer (900 µl) at a pH between 3–12 (in steps of 1 and at pH 5.5–9.5 in steps of 0.5) for 30 min on a rotary shaker (150 rpm). The experiment was continued as described in the general procedure and incubated at 25 °C. Buffers employed: pH 3–5: $Na_2HPO_4$–citric acid 50 mM; pH 7–9: Tris–HCl 50 mM; pH 10–12: phosphate 50 mM; pH 5.5–9.5: Bis-Tris–HCl 50 mM.

**3.1.1.3. Variation of concentration of ethanol.** As above using *trans*-anethole (6 µl, 40 µmol) as substrate, taking an appropriate amount of the supernatant (1 mg protein/ml) and an appropriate amount of ethanol.

**3.1.1.4. Variation of the substrate concentration.** In analogy to the procedure above but with 350 µl supernatant (1 mg protein/ml) and ethanol (150 µl). The amount of substrate and buffer chosen was appropriate to reach one milliliter of reaction mixture.

**3.1.1.5. Variation of the hydrogen peroxide concentration.** Experiments were performed as described in the general procedure. Different volumes of hydrogen peroxide (50–300 µl in steps of 50 µl) were used. In the case of different peroxide concentrations always the same volume of $H_2O_2$ (100 µl) was used but with different molarities (0.5–4 M hydrogen peroxide in steps of 0.5).

**3.1.1.6. GC-glass tubes.** Experiments were performed as described in the general procedure, but instead of Eppendorf tubes GC-glass vials (1.5 ml) were used, which were quickly closed with a rubber seal after addition of the hydrogen peroxide.

**3.1.2. Preparative alkene cleavage.** Lyophilized cells (3 g) of *T. hirsuta* G FCC 047 were rehydrated with Bis-Tris buffer (125 ml, 50 mM, pH 6) for 30 min on a rotary shaker (130 rpm, 25 °C). The mixture was transferred into the reaction vessel (450 ml) of 'Hydrogenation Apparatus Parr 3910' and *trans*-anethole **1a** (0.6 ml, 0.59 g, 3.9 mmol) and EtOH (1.7 ml) were added. The atmosphere was saturated with pure $O_2$ and then the oxygen pressure was adjusted to 2 bar. The oxygen was supplied from a 200 bar oxygen bottle. After 24 h of agitation at 22 °C under constant oxygen pressure (2 bar), the reaction mixture was extracted with EtOAc (4×50 ml) and centrifuged after each extraction step (8000 rpm, 20 min) to achieve phase separation. The cells were removed by filtration from the aqueous solution. The latter was once more extracted with EtOAc (50 ml). The combined organic phases were dried with $Na_2SO_4$, filtered and concentrated. A conversion of 81% *p*-anisaldehyde **2a** was observed by GC analysis. Column chromatography (50 g silica gel, petroleum ether/ethyl acetate=20:1) gave 0.31 g of *p*-anisaldehyde (57% isolated yield).

**3.1.2.1. Substrates.** All substrates (except **1b**) and reference aldehydes [except 2-(2-oxo-ethyl)-benzaldehyde and 2-(3-oxo-propyl)-benzaldehyde] were purchased from Aldrich, Lancaster and Acros with highest purity available. (*E*)-1-Phenyl-1-butene **1b** and 2-(2-oxo-ethyl)-benzaldehyde were synthesized as previously described.[16]

2-(3-Oxo-propyl)-benzaldehyde was synthesized from commercially available 1,2-dihydronaphthalene via its epoxide 1,2-epoxy-1,2,3,4-tetrahydro-naphthalene.

Powdered *m*-CPBA (1.03 mmol, 70%) was added to a stirred heterogeneous mixture of aqueous $NaHCO_3$ (0.3 N, 30 ml) and 1,2-dihydronaphthalene (0.76 mmol, 100 mg) at 4 °C over 15 min.[17] The suspension was vigorously stirred at 21 °C for 0.5 h and then extracted with ethyl ether (3×20 ml). The organic phase was washed with a cooled solution of 10% NaOH (20 ml), with brine (20 ml), dried over $Na_2SO_4$, and finally evaporated. Flash chromatography afforded 1,2-epoxy-1,2,3,4-tetrahydro-naphthalene (47 mg, 0.32 mmol, 43%) as colorless oil. $R_f$ (petroleum ether/EtOAc 10:1)=0.33; Structure was confirmed by comparison of NMR-data with literature.[18]

A solution of the 1,2-epoxy-1,2,3,4-tetrahydro-naphthalene (0.32 mmol, 47 mg) and ceric ammonium nitrate (0.21 mmol) in $CH_3CN$–$H_2O$ (3:1, 12 ml) was stirred at 21 °C for 1 h.[19] The solution was diluted with water (50 ml) and extracted with $CHCl_3$ (3×50 ml). The organic phase was dried over $Na_2SO_4$ and evaporated. Flash chromatography afforded pure dicarbonyl compound 2-(3-oxo-propyl)-benzaldehyde (11 mg, 22%) as colorless oil. $R_f$ (petroleum ether/EtOAc 1:1)=0.61; $^1$H NMR: 2.82 (t, 2H, $J$=7.3 Hz), 3.36 (t, 2H, $J$=7.3 Hz), 7.27–7.84 (m, 4H), 9.83 (s, 1H), 10.17 (s, 1H); $^{13}$C NMR: 25.7, 45.0, 127.1, 131.4, 133.9, 134.5, 142.7, 193.0, 201.2; *m/z*: 162, 144, 133, 118, 116, 115, 105, 91.

**3.1.2.2. Analytics.** GC-FID analyses were carried out on a Varian 3900 gas chromatograph equipped with FID and a HP 1701 capillary column (30 m, 0.25 mm, 0.25 μm film, $N_2$), 14.5 psi; temperature program: 100 °C–20 °C/min–220 °C—hold 2 min. Retention times: *trans*-anethole **1a** 4.44 min; *p*-anisaldehyde **2a** 4.75 min; (*E*)-1-phenyl-1-butene **1b** 3.31 min; benzaldehyde 2.97 min; indene **1e** 2.91 min; 2-(2-oxo-ethyl)-benzaldehyde 5.52 min; 1,2-dihydronaphthalene **1f** 3.65 min; 2-(3-oxo-propyl)-benzaldehyde 6.23 min; α-methyl styrene **1g** 2.54 min; acetophenone 3.34 min; thioanisole 3.95 min; aniline 3.60 min; *N*-methyl aniline 4.03 min.

GC–MS analyses were carried out on a Hewlett Packard 6890 equipped with FID and a HP Mass Selective Detector 5973. The GC was attached with a HP 5 MS capillary column (30 m, 0.25 mm, 0.25 μm film) and helium was used as carrier gas, the column head pressure was 9.65 psi; temperature program: 80 °C—hold 3 min–30 °C/min–250 °C—hold 10 min–30 °C/min–280 °C, 1 ml/min. Retention times: *trans*-anethole **1a** 6.61 min; *p*-anisaldehyde **2a** 6.44 min; (*E*)-1-phenyl-1-butene **1b** 5.53 min; benzaldehyde 4.03 min; 3-chloro styrene **1c** 5.11 min; 3-chloro benzaldehyde 5.53 min; styrene 3.24 min; *p*-amino styrene **1d** 6.3 min; *p*-amino benzaldehyde 7.3 min; indene **1e** 4.80 min; 2-(2-oxo-ethyl)-benzaldehyde 6.90 min; 1,2-dihydronaphthalene **1f** 5.88 min; 2-(3-oxo-propyl)-benzaldehyde 7.40 min; α-methyl styrene **1g** 4.23 min; acetophenone 5.05 min.

## Acknowledgements

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