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Biotransformation of diphenyl ether by the yeast *Trichosporon beigelii* SBUG 752

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Abstract

Trichosporon beigelii SBUG 752 was able to transform diphenyl ether. By TLC, HPLC, GC, GC-MS, NMR- and UV-spectroscopy, several oxidation products were identified. The primary attack was initiated by a monooxygenation step, resulting in the formation of 4-hydroxydiphenyl ether, 2-hydroxydiphenyl ether and 3-hydroxydiphenyl ether (48:47:5). Further oxidation led to 3,4-dihydroxydiphenyl ether. As a characteristic product resulting from the cleavage of an aromatic ring, the lactone of 2-hydroxy-4-phenoxymuconic acid was identified. The possible mechanism of ring cleavage to yield this metabolite is discussed.

Introduction

During the combustion of industrial and municipal wastes and in the course of several syntheses of chemicals, especially of chlorinated compounds, polychlorinated dibenzo-p-dioxins and dibenzofurans are formed (Hutzinger 1985). These widespread compounds are persistent environmental pollutants showing an acute toxicity (Plüss et al. 1988) and high resistance to microbial attack. Therefore, it is of interest to search for microorganisms able to degrade these pollutants. Because of the high toxicity of the chlorinated compounds, unchlorinated dibenzo-p-dioxin, dibenzofuran or diphenyl ether are sometimes used as substrates in principle investigations (Strubel et al. 1989; Fortnagel et al. 1989).

In the yeast *Trichosporon beigelii* SBUG 752, diphenyl ether (DPE) and other aromatic substances are oxidized to a remarkable degree (Schauer et al. 1993). However, no considerable growth on this substrate could be observed.

In this paper we describe the first steps in the degradation pathway characterized by chemical analysis of the oxidation products formed in *Trichosporon beigelii* SBUG 752 after incubation with DPE.

Methods and materials

Microorganism, growth and incubation conditions

Trichosporon beigelii SBUG 752 (Schauer et al. 1993) was isolated from soil contaminated with car exhaust. The strain was cultivated on malt agar slants. For degradation experiments the yeast cells were propagated in shake flasks, which contains 100 ml of a mineral salts medium supplemented with 1 g D-glucose as carbon source and 1 ml of vitamin solution (Schauer et al. 1993). After incubation for 24 h at 30° C on a rotary shaker, the cells were harvested by centrifugation (6000 × g) and washed with sterile phosphate buffer (0.05 M, pH 5.5). The pellet (about 0.6 g wet weight) was resuspended in 50 ml sterilized mineral salts medium. As substrate, 3–15 μl (29–147 μmol/g dry weight) DPE was added. Then the shake flasks were again incubated for different times as described

above. Control experiments were performed both with flasks which contained mineral salts solution and cells without DPE and with mineral salts solution and DPE without cells. At the end of the incubation time, the cultures were examined microscopically and by plating for purity to exclude microbial contaminants.

Extraction

After incubation with DPE the cells were harvested by centrifugation and the supernatant (about 50 ml) was extracted three times with 50 ml of diethylether at pH 5.5, yielding the 'neutral extract'. Subsequently the culture fluid was acidified to pH 2 with hydrochloric acid and extracted again to yield the 'acid extract'. The extracts were dried over anhydrous sodium sulphate, the solvent was removed and the residues were dissolved in 100 μl of acetone or methanol. These extracts were examined for metabolic degradation products by thin layer chromatography, HPLC, GC, GC-MS, NMR- and UV-spectroscopy.

Analytical methods

Thin layer chromatography (TLC) was carried out with Serva Silufol sheets UV 254 with fluorescent indicator. The solvent system was benzene-dioxane-acetic acid (90:25:4; v:v:v). Compounds were visualized by UV-light and by spraying with a 10% ethanolic molybdatophosphoric acid solution or a solution of diazotated benzidine (Stahl 1967).

High performance liquid chromatography (HPLC) was used for detection of metabolites in the supernatant of culture fluid without previous solvent extraction. The system (Hewlett Packard GmbH, Bad Homburg, FRG) consisted of a HP 1050 Series Pumping System, a HP 1040 M Series I Diode-Array-Detector and a HP HPLC Chem Station. A LiChroCart 125-4 RP 18 endcapped (5 μm) column (Merck, Darmstadt, FRG) was used. Separation was achieved with a solvent system of methanol and water (containing 1 g 99% phosphoric acid per 1 l) with a gradient from 40% to 55% methanol. The flow rate was 1 ml/min. The metabolites were detected by measuring the absorption at 210 nm and 254 nm and identified by their retention times and in situ UV spectra. They were compared with those of available authentic compounds by using a spectra library.

Gas chromatography (GC) was carried out in a GC-M Chromatograph (Beckman Instruments GmbH, Munich, FRG) with a flame ionization detector (275° C) at a temperature program from 50° C to 250° C (5° C/min) and a flow rate of 18.3 ml/min. It was equipped with a packed column (180 × 0.3 cm), Silicon JxR, 3% (Serva) on Gaschrom Q (130–150 μm). Argon was used as the carrier gas. Methyl esters of acid metabolites were prepared with BF_3/methanol.

For *Gas chromatography/Mass spectroscopy* (GC-MS) analyses, a coupling system was used consisting of a HP 5890 gas chromatograph (Hewlett Packard GmbH, Bad Homburg, FRG) linked to a VG 70–250 S mass spectrometer (Vacuum Generators, Manchester, UK) operating at 70 eV. The separation was carried out on a 50 m CP-Sil 8 fused silica column (Chrompack GmbH, Frankfurt, FRG) with a temperature program from 80 to 300° C at a rate of 5° C/min. As carrier gas Helium was used with a flow rate of 1.9 ml/min.

UV spectra were determined on a Uvikon 930 spectrophotometer (Kontron Instruments, Eching, FRG).

Proton magnetic resonance spectra were obtained with a Bruker WM 400 instrument (Bruker GmbH, Karlsruhe, FRG).

Chemicals

Diphenyl ether (> 98%), 3-hydroxydiphenyl ether (98%) and 4-hydroxydiphenyl ether (99%) were purchased from Aldrich-Chemie, Steinheim, FRG. 2-HydroxyDPE was prepared from guaiacol and bromobenzene via 2-methoxydiphenyl ether (Ungnade et al. 1946, 1950). 3,4-Dihydroxydiphenyl ether was prepared from 4-bromoveratrole and phenol via 3,4-dimethoxydiphenyl ether (Janssen et al. 1955; Mc Omie & West 1969). Borontrifluoride (14% in methanol) was purchased from Serva, Heidelberg, FRG. Diethylether was distilled (after addition of 90 ml aqua dest., 22.5 g $FeSO_4$ and 2.25 g CaO per l) to remove peroxides. All other chemicals and solvents were of the highest purity available.

Results

To identify metabolites, yeast cells incubated for 24 h with DPE were harvested by centrifugation and the culture fluid was extracted to obtain a neutral and an acid extract. In addition to unoxidized DPE, TLC analysis of these extracts revealed the presence of three compounds (compounds I, II and III) in the neutral extract and a main component (IV) in the acid fraction (see

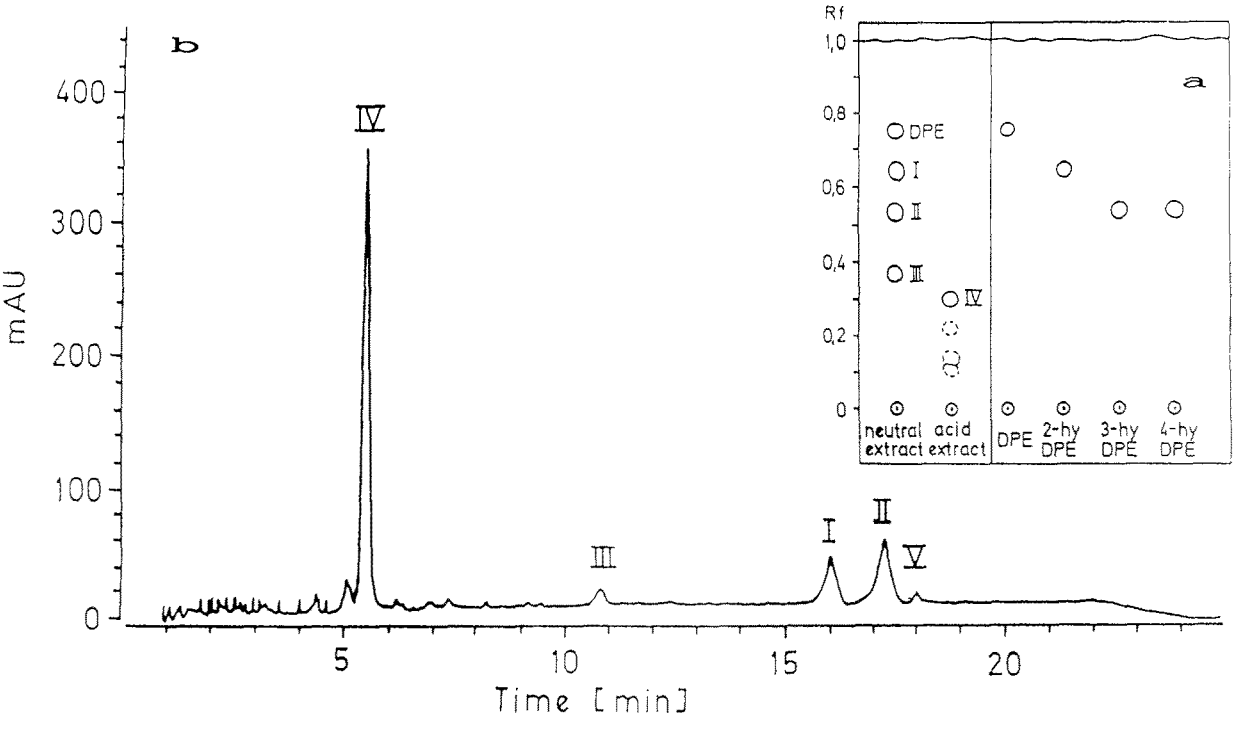

Fig. 1. TLC analysis (a) and HPLC elution profile (b) of metabolites formed from DPE in *Trichosporon beigelii*. For HPLC analysis samples of 1 ml were removed from culture fluid and injected after harvesting cells by centrifugation. HPLC conditions were as described in Methods. (I) 2-hydroxyDPE, (II) 4-hydroxyDPE, (III) 3,4-dihydroxyDPE, (IV) 6-carboxy-4-phenoxy-2-pyrone (the lactone of 2-hydroxy-4-phenoxymuconic acid), (V) 3-hydroxydiphenyl ether; hy-DPE = hydroxydiphenyl ether.

Fig. 1). The products were not found in extracts from control experiments without cells or without DPE.

Identification of compounds in the neutral extract

Compound I (Rf = 0.64) and compound II (Rf = 0.53) absorbed UV light, gave a black colour in TLC when sprayed with molybdatophosphoric acid and a brown colour with diazotated benzidine, pointing towards the presence of a phenolic hydroxy group. The metabolites were separated by preparative TLC. The UV light absorbing spots were scraped off and extracted with diethylether. The UV absorption spectra of compounds I and II showed absorption maxima at 272 nm, 278 nm and 282 nm with a shoulder at 291 nm. The mass spectra of both compounds, obtained by GC-MS analyses, showed molecular ions at m/z 186, which is in accordance with a molecular formula of $C_{12}H_{10}O_2$, and fragment ions at m/z 169 (loss of OH), 157, 129, 128, 109 (loss of $C_6H_5^+$), 77 ($C_6H_5^+$), 51 and 39. The molecular formula $C_{12}H_{10}O_2$ corresponds to monohydroxydiphenyl ether.

Comparative analysis by TLC, UV-spectroscopy, GC and GC-MS verified the identity of compound I with 2-hydroxydiphenyl ether. This is supported by the identification of peak I (t_R = 16.03 min) in HPLC analysis as 2-hydroxydiphenyl ether.

Compound II showed the same Rf-value and colour reaction in TLC, UV absorption maxima and retention times in GC analysis as 3- or 4-hydroxydiphenyl ether. Since mass spectra of 3- and 4-hydroxydiphenyl ether can be distinguished (McLafferty & Stauffer 1989), our GC-MS analysis suggested that compound II may be represented by a mixture of 3- and 4-hydroxydiphenyl ether, the latter forming the major product. This was supported by HPLC analysis of the culture fluid (see Fig. 1) which showed that peaks II and V with t_R = 17.2 min and 18.3 min, are identical with 4- and 3-hydroxydiphenyl ether, respectively. Thus the detection of hydroxydiphenyl ether by HPLC agreed with results from previous analyses. 3-Hydroxydiphenyl ether appeared only in a small amount. Because it was not possible to separate 3- and 4-hydroxydiphenyl ether by TLC and GC (under the chosen conditions), 3-

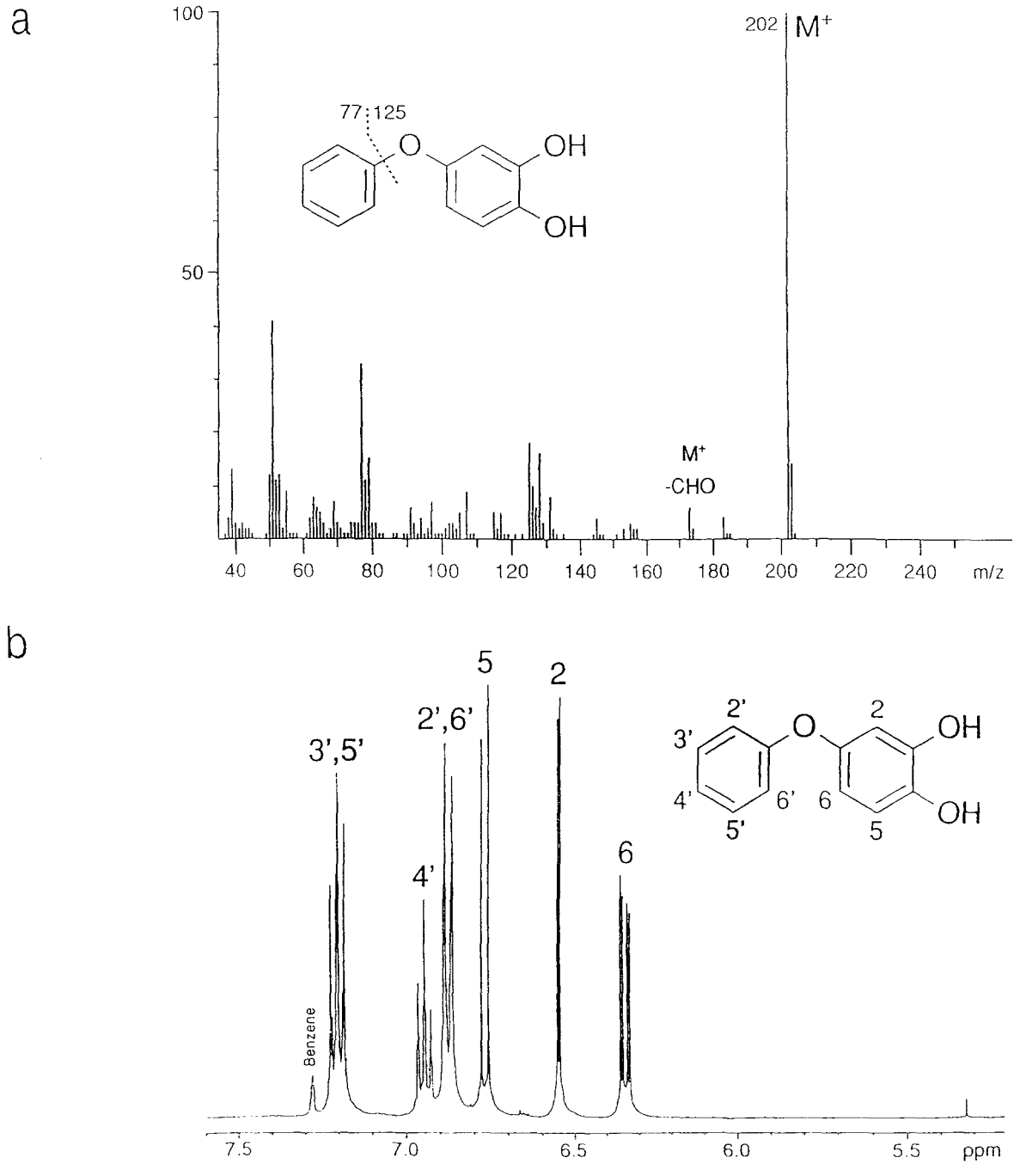

Fig. 2. Mass spectrum (a) of extracted 3,4-dihydroxydephenyl ether and ^{1}H NMR spectrum (b) of authentic synthesized 3,4-dihydroxydiphenyl ether. 400 MHz; CD_3OD; TMS was the internal standard.

hydroxydiphenyl ether might have been hidden in such analyses by the large amount of 4-hydroxydiphenyl ether.

Compound III (Rf = 0.36) also revealed a brown colour in TLC when sprayed with diazotated benzidine indicating the existence of aromatic hydroxy groups. In HPLC analysis compound III had a retention time of 10.77 min. The mass spectrum obtained by GC-MS analysis (see Fig. 2a) showed a molecular ion peak at m/z 202, which corresponds to dihydrox-

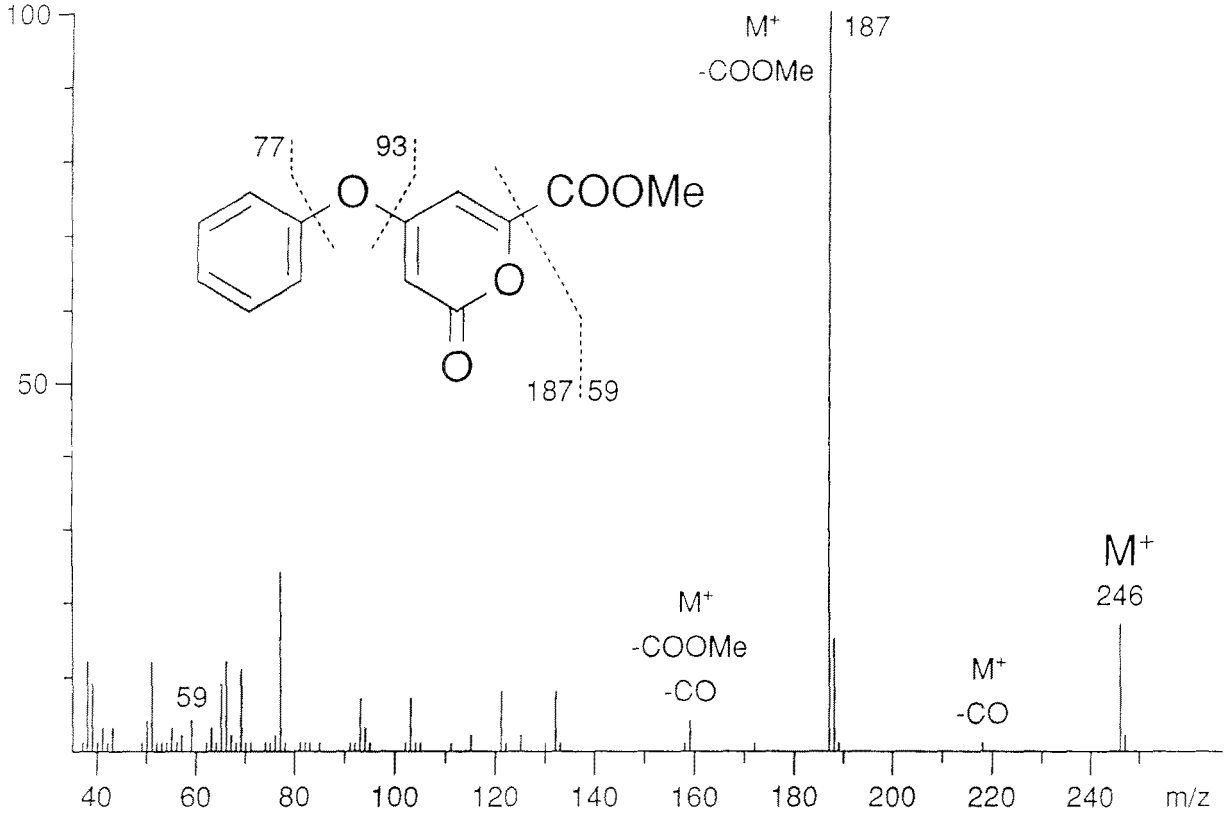

Fig. 3. Mass spectrum of compound IV, 6-carboxy-4-phenoxy-2-pyrone.

ydiphenyl ether. Important ion peaks were m/z 173 (M^+-CHO), 125 (M^+-C_6H_5) and 77 ($C_6H_5^+$). Both HPLC and MS data correspond to those of authentic 3,4-dihydroxydiphenyl ether. Evidence for the structure of the synthesized compound was the ^{1}H NMR spectrum (Fig. 2b). Data were as follows: δ = 6.34 (dd, 1 H, 6-H), 6.55 (d, 1 H, 2-H), 6.77 (d, 1 H, 5-H), 6.85–6.90 (m, 2 H, 2′,6′-H), 6.92–6.97 (m, 1 H, 4′-H), 7.17–7.24 (m, 2 H, 3′,5′-H) ppm. $J_{2,6}$ = 2.8 Hz, $J_{5,6}$ = 8.6 Hz.

Identification of compounds in the acid extract

Compound IV had a Rf value of 0.3 and absorbed UV light in TLC. In the UV spectrum absorption maxima were determined at 207 nm and 295 nm. For GC analysis, methylation of extracted compounds was necessary. The mass spectrum of the methylated compound IV obtained by GC-MS analysis is given in Fig. 3. We found the molecular ion peak at m/z 246 and diagnostically important ion peaks at m/z 218 (M-CO), 187 (M-$COOCH_3$), 159 (M$COOCH_3$-CO), 93 (C_6H_5O), 77 (C_6H_5) and 59 ($COOCH_3$). According to the molecular ion peak m/z 246, the molecular formula is $C_{13}H_{10}O_5$, from the corresponding unmethylated acid $C_{12}H_8O_5$. The ^{1}H NMR data (400 MHz, CD_3OD, tetramethylsilane (TMS) as internal standard), (see Fig. 4) obtained after separation of the acid extract by preparative HPLC, were as follows: δ = 5.31 (d, 1 H, 3-H), 6.90 (d, 1 H, 5-H), 7.14–7.18 (m 2 H, 2′,6′-H), 7.3–7.36 (m, 1 H, 4′-H), 7.45–7.51 (m, 2 H, 3′,5′-H) ppm. $J_{3,5}$ = 2.3 Hz. These data are consistent with the lactone of the 2-hydroxy-4-phenoxy-muconic acid (6-carboxy-4-phenoxy-2-pyrone).

Additional minor peaks in HPLC analyses have not been identified so far.

Incubation of cells with 2-, 3- and 4-hydroxydiphenyl ether

To confirm the degradation pathway of DPE, 2-, 3- and 4-hydroxydiphenyl ether were examined for oxidation by *Trichosporon beigelii* SBUG 752. Glucose grown cells were incubated in the presence

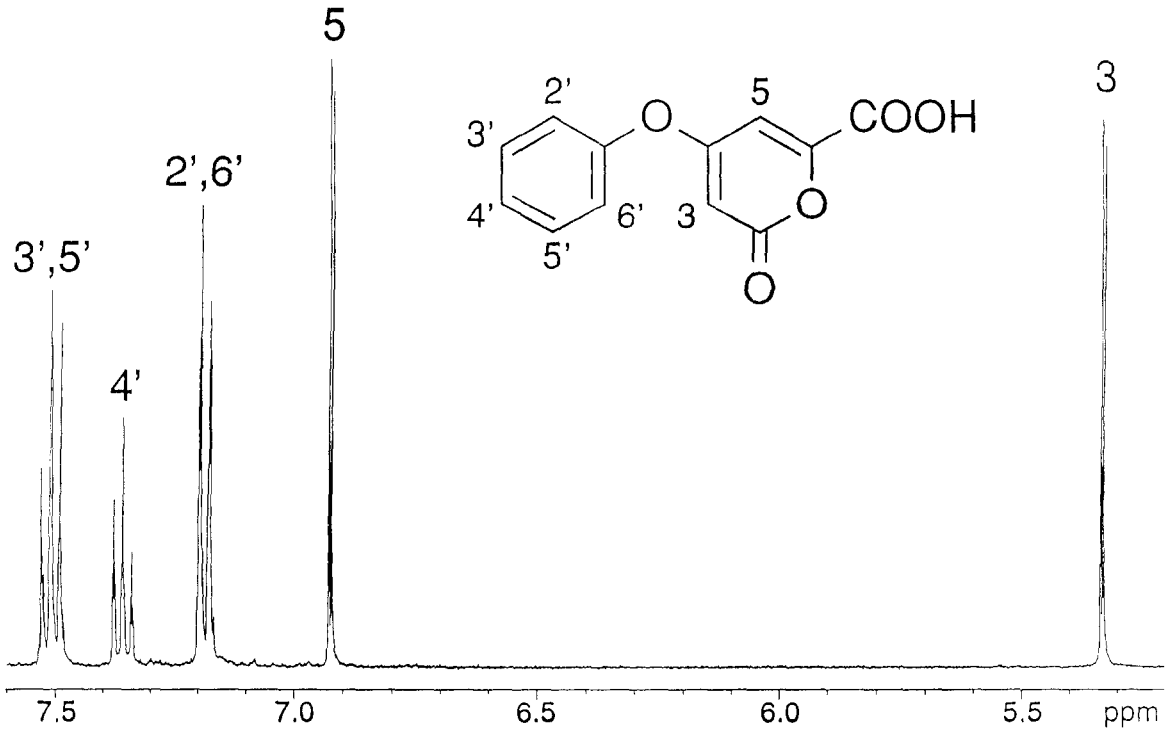

Fig. 4. ^{1}H NMR spectrum of compound IV, the lactone of 2-hydroxy-4-phenoxymuconic acid formed during oxidation of DPE in *Trichosporon beigelii* (400 MHz; CD_3OD); TMS was the internal standard.

of these compounds as substrates and the formation of metabolites was monitored by HPLC. Analyses revealed that 3- and 4-hydroxydiphenyl ether were oxydized to the major products 3,4-dihydroxydiphenyl ether and 6-carboxy-4-phenoxy-2-pyrone, as already demonstrated with DPE as substrate. In the case of 3-hydroxydiphenyl ether, smaller amounts of these products were accumulated. As expected, after incubation with 2-hydroxydiphenyl ether these metabolites would not be found.

Discussion

The degradation of aromatic compounds is a well known property of many yeasts. Especially the utilization of phenol was intensively studied in *Trichosporon cutaneum* (new nomenclature: *T. beigelii*) and this capability seems widely distributed among yeasts (Gaal & Neujahr 1980; Hofmann & Schauer 1988). However, the degradation of diaryl compounds by yeasts or filamentous fungi has received less attention up to now. For *Saccharomyces cerevisiae*, the formation of 4-hydroxybiphenyl from biphenyl was described by Wiseman et al. (1975). Cerniglia & Crow (1981) showed the oxidation of biphenyl to 2-, 3-, and 4-hydroxybiphenyl, 4,4'-dihydroxybiphenyl and 3-methoxy-4-hydroxybiphenyl by *Candida lipolytica*. Dodge et al. (1979) also reported on the oxidation of biphenyl by *Cunninghamella elegans*. Seigle-Murandi et al. (1991) reported that 4-hydroxydiphenyl ether is primarily formed from DPE in the filamentous fungus *Cunninghamella echinulata*. Cerniglia et al. (1979) investigated the oxidation of dibenzofuran by bacteria and fungi. The results reported here indicate that *Trichosporon beigelii* SBUG 752 has the ability to metabolize DPE. The aim of the present study was the identification of some typical metabolites. According to the results obtained, we propose the pathway for oxidation by *Trichosporon beigelii* in Fig. 5. The hydroxylation occurs at all three positions, 2-, 3-, and 4-hydroxydiphenyl ether are accumulated in a ratio of 47:5:48. It may be possible that these comppounds are formed by spontaneous isomerization of related arene oxides, as described by Cerniglia & Gibson (1977) for the oxidation of naphthalene and antracene by *Cun-*

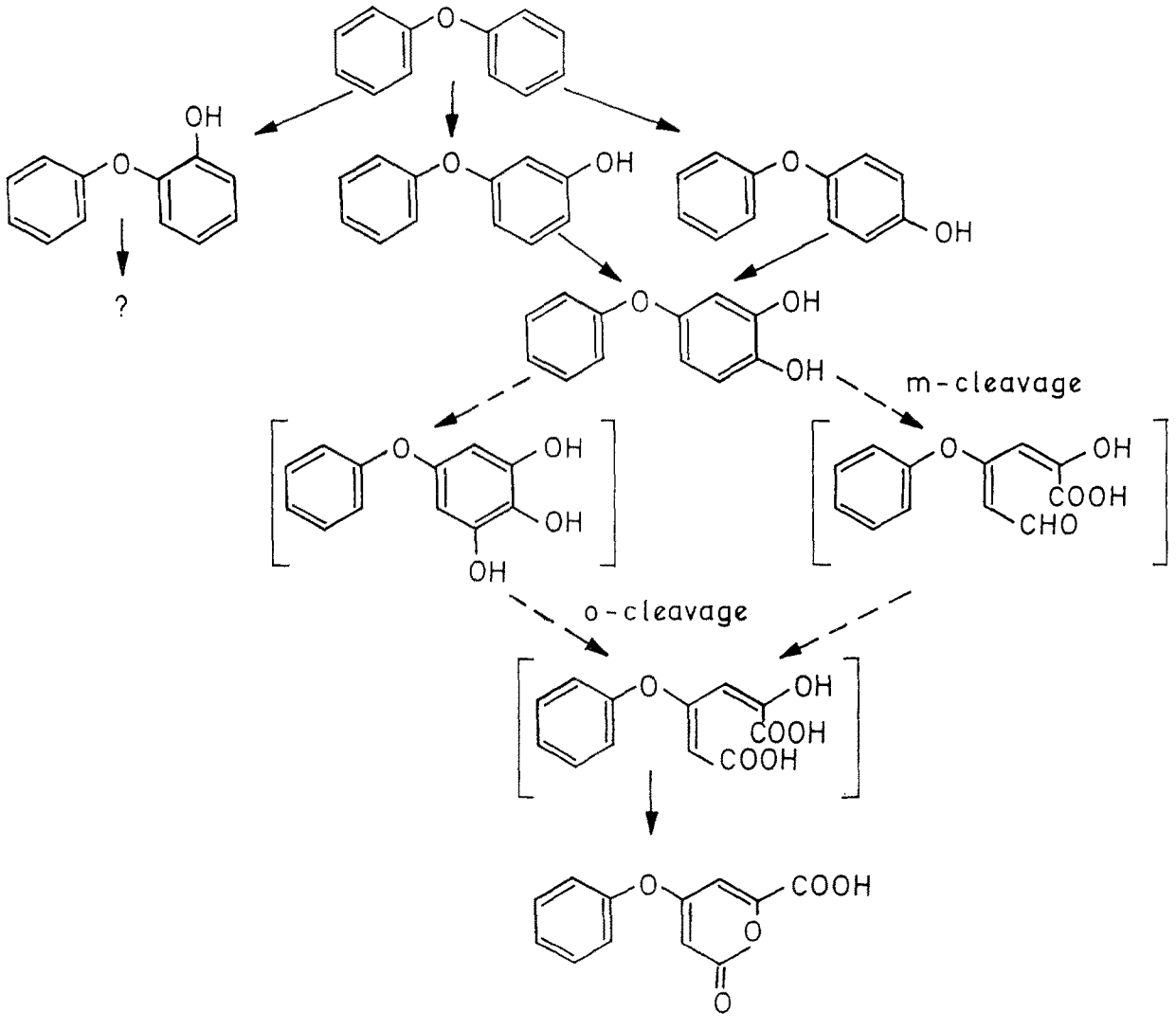

Fig. 5. Proposed pathway for the oxidation of DPE in *Trichosporon beigelii* SBUG 752.

ninghamella elegans. Daly & Jerina (1972) showed the formation of arene oxides during the metabolism of aromatic substances in biological systems by the NIH shift. Cerniglia (1981) also reported on the formation of hydroxylated isomers via the 2,3-oxide and 3,4-oxide for the oxidation of biphenyl and *Cunninghamella elegans*. Synthetic biphenyl-3,4-oxide isomerizes exclusively to 4-hydroxy-biphenyl (Cerniglia 1981) and investigations of Servé & Jerina (1978) indicated the formation of 2- and 3-hydroxybiphenyl in a ratio of 49:1 by rapid isomerization of biphenyl-2,3-oxide.

These results would explain the observed ratio of 2-, 3- and 4-hydroxydiphenyl ether. However, we could not detect these arene oxides in the culture medium of *Trichosporon beigelii* SBUG 752.

In addition to the monohydroxylated compounds we identified 3,4-dihydroxydiphenyl ether by TLC, HPLC, GC-MS and UV spectroscopy.

Concerning the oxidation of diaryl compounds by yeasts or filamentous fungi, the formation of hydroxylated products has been described by several authors (see above). But as far as we know, no report on ring cleavage of such compounds exists. Our results show that the lactone of 2-hydroxy-4-phenoxymuconic acid is formed during the degradation of DPE by *Trichosporon beigelii* SBUG 752. This lactone should be formed from the corresponding acid. Gurujeyalaksh-

mi & Mahadevan (1987) reported that in a comparable reaction gallic acid is degraded to 2-hydroxy-*cis, cis*-muconic acid by *Aspergillus flavus*. The possibility of spontaneous lactonization of substituted 2-hydroxymuconic acid to the corresponding lactone has been described by Eaton & Ribbons (1982, 1987).

In general, eucaryotic microorganisms use ortho-fission to degrade the aromatic ring system. An exception was reported by Walker & Taylor (1983). They reported on a cleavage between two hydroxy groups during the metabolism of pyrogallol by *Fusarium solani*, which they called meta-fission because of the formation of pyruvate via oxalocrotonate.

Trichosporon cutaneum degrades phenol via catechol by an ortho-fission pathway (Spanning & Neujahr 1987; Gaal & Neujahr 1980). *Trichosporon beigelii* SBUG 752 also degraded phenol via an ortho-fission pathway (unpublished results). The formation of 2-hydroxy-4-phenoxymuconic acid takes place either by meta-fission of 3,4-dihydroxydiphenyl ether or by ortho-fission of trihydroxylated DPE. The latter compound, however, has not been detected as an intermediate as yet.

We found pyrogallol as an intermediate in the degradation of phenol by *Trichosporon beigelii* SBUG 752. According to this and the reports mentioned above, an ortho-fission of 3,4,5-trihydroxydiphenyl ether seems more likely. Other parallel degradation pathways (e.g. the direct ortho- fission of 3,4-dihydroxydiphenyl ether) without accumulation of detectable amounts of oxidation products under the given incubation conditions may be possible.

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