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NOVEL CHLOROMETABOLITES PRODUCED BY *BJERKANDERA* SPECIES

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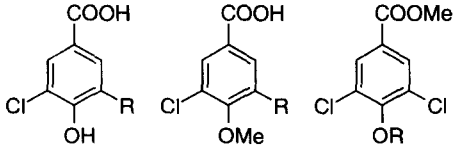
Key Word Index—*Bjerkandera* sp. strain BOS55; *Bjerkandera adusta*; Basidiomycetes; novel chlorometabolites; chlorinated benzoic acid derivatives; potential chlorophenol precursors.

Abstract—The EtOAc extract from the extracellular fluid of the mycelium of *Bjerkandera* sp. BOS55 contained four novel chlorinated benzoic acid derivatives, i.e. 3-chloro-4-hydroxybenzoic acid, 3,5-dichloro-4-hydroxybenzoic acid, methyl 3,5-dichloro-4-hydroxybenzoate and methyl 3,5-dichloro-*p*-anisate. 3-Chloro-4-hydroxybenzoic acid was also produced by *B. adusta*.

INTRODUCTION

Halogenated aromatic compounds cannot only be regarded as xenobiotic compounds from industrial sources, but also as natural products [1]. Although the majority of the natural organohalogens are metabolites from organisms living in marine environments, several strains of different genera of wood- and forest litter-degrading basidiomycetes are also known to produce halogenated compounds [2].

Chlorinated anisyl metabolites (CAM) are the most common organohalogens produced by basidiomycetes. To date, 12 different genera were reported to produce CAM [3]. White-rot fungi belonging to the genus *Bjerkandera*, for instance, are known to produce *de novo* the following CAMs: 3-chloro-*p*-anisaldehyde (CAld) and 3,5-dichloro-*p*-anisaldehyde (DCAld) and the corresponding chlorinated anisyl alcohols in concentrations up to 47 mg l^{-1} [4–7]. Both chloro- and 2,6-dichloro-1,4-dimethoxybenzene have also been detected but at much lower concentrations [6]. In the present paper we report the *de novo* biosynthesis of six other chlorinated metabolites by fungal strains of the genus *Bjerkandera*. The new metabolites are composed of chlorinated 4-hydroxybenzoic acids or their methyl ether and/or methyl ester derivatives. Four of these compounds were not previously reported as *de novo* metabolites, from basidiomycetes or any other living organism.



1: R = H **2**: R = Cl **3**: R = H **4**: R = Cl **5**: R = H **6**: R = Me

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RESULTS AND DISCUSSION

The fungal strains (*Bjerkandera* sp. BOS55 and *B. adusta*) were cultivated in the dark at 25° on a high-nitrogen-content peptone medium [8]. When the culture fluid was completely covered by the mycelium (3–4 weeks), the culture fluid was filtered and extracted with EtOAc (see Experimental). The identification of chlorinated compounds in the EtOAc extract was performed with GC-mass spectrometry, and comparison of retention times and mass spectra to data of respective reference compounds, mostly commercially available. The novel chlorinated compounds found in the EtOAc extracts from *Bjerkandera* sp. BOS55 and *B. adusta* are listed in Table 1.

The EtOAc extract of the extracellular fluid of the mycelium of *Bjerkandera* sp. BOS55 appeared to contain a considerable number of chlorinated organic compounds of which nine could be analysed. Three of these compounds, i.e. the two chlorinated *p*-anisaldehydes (CAld and DCAld) and chloro-1,4-dimethoxybenzene (CDMB), were known metabolites from *Bjerkandera*. Two other compounds could be characterized as 3-chloro-*p*-anisic acid (**3**) and 3,5-dichloro-*p*-anisic acid (**4**). 3-Chloro-*p*-anisic acid was the most abundant metabolite of these two compounds. Both acids were recently detected in the soil collected at the arch of a *Lepista nuda* fairy ring growing in a coniferous forest [9]. The production of **3** and **4** by *Bjerkandera* sp. BOS55, however, has not been reported before. The remaining four metabolites could be di-

Table 1. GC-MS determination of novel chlorinated compounds from *Bjerkandera* spp.

Compound	*Bjerkandera* spp.	
	BOS55	BEUK47
1	+	+
2	+	−
3 + **4***	+++	+++++
5	++	−
6	+	−
CAld†	+++	++
DCAld†	+	+
CDMB†	+	−

BOS55, *Bjerkandera* sp. strain BOS55; BEUK47, *B. adusta* strain BEUK47; CAld, 3-chloro-*p*-anisylaldehyde; DCAld, 3,5-dichloro-*p*-anisylaldehyde; CDMB, 2-chloro-1,4-dimethoxybenzene; Symbols: − not detected, + ≤ 1%, ++ ≤ 5%, +++ ≤ 10%, +++++ > 50%. Percentages refer to total detected chlorinated and non-chlorinated metabolites.

*Partly overlapped peaks in GC-MS, [**3**]>> [**4**].

†Known compounds from *Bjerkandera* spp.

vided into two groups, chlorinated 4-hydroxybenzoic acids and methyl esters of dichlorinated benzoic acids. The first group consists of 3-chloro-4-hydroxybenzoic acid (**1**) and 3,5-dichloro-4-hydroxybenzoic acid (**2**), and the second group consists of methyl 3,5-dichloro-4-hydroxybenzoate (**5**) and methyl 3,5-dichloro-*p*-anisate (**6**). As far as we know this is the first report of the *de novo* biosynthesis of these compounds by a fungus or any other living organism. In particular, the *de novo* biosynthesis of the chlorinated 4-hydroxybenzoic acids **1** and **2** is of interest. It is known that these acids can undergo facile decarboxylation to the corresponding chlorophenols under basic conditions [10]. Anaerobic bacteria are also known for their abilities to convert these compounds into chlorophenols [11, 12]. It is therefore not excluded that **1** and **2** can serve as natural precursors for chlorophenols which have been classified as priority pollutants [13]. The chlorophenols in turn can give rise to the formation of chlorinated dibenzo-*p*-dioxins and dibenzofurans via peroxidase-mediated oxidation processes [14–16].

In comparison with *Bjerkandera* sp. BOS55, the number of different chlorinated compounds in the EtOAc extract of *B. adusta* was distinctly lower. Only four known compounds (**3**, **4**, CAld and DCAld) and the novel benzoic acid **1** could be detected. On the other hand, the relatively high production of **3** (>50% of total compounds) by this fungal strain is noticeable. Bacterial or fungal *O*-demethylation [17] of **3** (or **4**) followed by decarboxylation might also result in chlorophenol formation.

The occurrence of chloro-4-hydroxybenzoic acids (**1** and **2**), chloro-*p*-anisic acids (**3** and **4**) as well as chlorinated methyl esters (**5** and **6**) in *Bjerkandera* and their structural similarities suggest that these compounds are biosynthetically related to each other. Methylation of the 4-hydroxy group by intracellular *O*-methyltransferases utilizing either *S*-adenosylmethionine or chloromethane as a physiological methyl donor is well described in white rot fungi [18–20]. Esterification of benzoic acids to the corresponding methyl esters which could potentially account for the production of **5** and **6** from **2** and **4**, respectively, has also been reported [21].

EXPERIMENTAL

General. Mps: uncorr.; ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) in $CDCl_3$, δ in ppm (int. standard: TMS).

Organisms and culture conditions. Bjerkandera sp. strain BOS55 and *B. adusta* strain BEUK47 were obtained from the Culture Collection of Industrial Microbiology (CIMW), Agricultural University Wageningen, The Netherlands.

Both fungal strains were grown on a high-nitrogen-content medium (50 mM N) supplied as peptone containing (g l^{-1}): glucose · H_2O, 20.00; mycological peptone (Oxoid Ltd, Basingstoke, Hampshire, UK), 5.00; yeast extract (Gibco BRL, Life Technol. Ltd, Paisley, Scotland, U.K.), 2.00; KH_2PO_4, 1.00; $MgSO_4 \cdot 7H_2O$, 0.50; NaCl, 0.06 [8]. Medium (10 ml liquid vol.) in a 100 ml serum bottle was sterilized at 121° for 30 min. Medium was inoculated with a plug (diameter 5 mm), which was taken from an agar medium covered with mycelium of the fungal strain. Fungal cultures were incubated in the dark at 25° under air. When the culture fluid was completely covered by the mycelium (3–4 weeks), the culture fluid was harvested.

Extraction and sample prepn. After filtration of the extracellular fluid, pH of the filtrate was adjusted to 2 with 4 M H_2SO_4 followed by extraction 3× with freshly distd EtOAc. The combined organic layers were washed with H_2O and concd under red. pres. at ambient temp. The concentrate was filtered over silica gel 60 (230–400 mesh) (Merck) using freshly distd EtOAc as the eluent. After removal of the solvent under red. pres., the remaining residue was redissolved in 0.5 ml of freshly distd EtOAc and then subjected to GC-MS analysis.

Identification and quantitation of chlorometabolites by GC-MS. All the samples were analysed on an HP5970B quadrupole MS coupled to an HP5890 gas chromatograph equipped with a fused silica capillary column (DB17, 30 m × 0.25 mm i.d., film thickness: 0.25 μm). Carrier gas and flow: He at 1.1 ml min^{-1}. Injector temp. 220°; temp. programme: 70–250° at 7° min^{-1}, hold 20 min. Injection vol.: 10 μl; split ratio 1:100. EIMS were obtained at 70 eV, and quantitation was based on the total ion current which gave an indication of the relative concn of the compound in the extract. The identification of the chlorinated compounds was achieved by comparison of R_ts and MS to data of respective authentic compounds. All measurements were done in duplicate from a duplicate set of cultures.

Authentic compounds. 3-Chloro-4-hydroxybenzoic acid (**1**), 3,5-dichloro-4-hydroxybenzoic acid (**2**), 3-chloro-*p*-anisic acid (**3**) and methyl 3,5-dichloro-4-hydroxybenzoate (**5**) were commercially available from Lancaster (Mühlheim am Main, Germany). Methyl 3,5-dichloro-*p*-anisate (**6**) was prepd from **2** as described [7].

Preparation of 3,5-*dichloro*-p-*anisic acid* (**4**). Ester **6** was saponified with KOH in aq. MeOH to give 3,5-dichloro-*p*-anisic acid (**4**, in 70% after crystallization from EtOAc–petrol). White powder, mp 163–165°. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.96 (*s*, 3H, MeO-4), 7.97 (*s*, 2H, C-2 and C-6). ^{13}C NMR (50 MHz, $CDCl_3$): δ 61.4 (*q*, MeO-4), 129.3 (*s*, C-1), 130.2 (2*s*, C-3 and C-5), 131.2 (2*d*, C-2 and C-6), 156.8 (*s*, C-4), 165.1 (*s*, CO).

MS. The spectra of the six novel halometabolites from *Bjerkandera* spp. (**1**–**6**) are shown below; only peaks with intensities higher than 10% are given.

3-*Chloro*-4-*hydroxybenzoic acid* (**1**). *m/z* rel. int.): 174 $[M+2]^+$ (20), 172 $[M]^+$ (55), 157 (35), 155 (100), 127 (22), 99 (30), 91 (18), 73 (30), 63 (76), 62 (40), 61 (22), 53 (37), 45 (30).

3,5-*Dichloro*-4-*hydroxybenzoic acid* (**2**). *m/z* (rel. int.): 208 $[M+2]^+$ (51), 206 $[M]^+$ (72), 191 (63), 189 (100), 133 (18), 125 (17), 97 (24), 73 (22), 63 (33), 62 (47), 61 (31), 53 (23), 45 (17).

3-*Chloro*-p-*anisic acid* (**3**). *m/z* (rel. int.): 188 $[M+2]^+$ (35), 186 $[M]^+$ (100), 171 (31), 169 (67), 115 (28), 63 (33), 51 (16).

3,5-*Dichloro*-p-*anisic acid* (**4**). *m/z* (rel. int.): 224 $[M+4]^+$ (10), 222 $[M+2]^+$ (65), 220 $[M]^+$ (100), 207 (29), 205 (56), 177 (17), 149 (45), 97 (44), 74 (32), 62 (35).

Methyl 3,5-*dichloro*-4-*hydroxybenzoate* (**5**). *m/z* (rel. int.): 222 $[M+2]^+$ (26), 220 $[M]^+$ (38), 193 (16), 192 (18), 191 (79), 190 (28), 189 (100), 161 (16), 135 (15), 133 (24), 125 (20), 97 (32), 73 (22), 63 (23), 62 (44), 61 (25), 53 (17).

Methyl 3,5-*dichloro*-p-*anisate* (**6**). *m/z* (rel. int.): 236 $[M+2]^+$ (32), 234 $[M]^+$ (46), 205 (73), 203 (100), 111 (25), 109 (16), 99 (21), 97 (49), 75 (20), 74 (26), 62 (29), 61 (18).

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