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THE FORMATION OF VERATRYL CHLORIDE BY *BJERKANDERA* SP. STRAIN BOS55

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Key Word Index—*Bjerkandera* sp. strain BOS55; white-rot fungus; novel chlorometabolite; veratryl chloride.

Abstract—In the ethyl acetate extract from the extracellular fluid of the mycelium of *Bjerkandera* sp. BOS55 the presence of veratryl chloride was detected. Addition of deuterated benzoic acid or 4-hydroxybenzoic acid to the culture resulted in the formation of deuterated veratryl chloride. The detection of veratryl chloride constitutes the first report of a biologically derived natural product which contains a halogenated benzylic carbon. Since veratryl chloride is not very stable in aqueous medium, its detection also suggests a continuous production. © 1997 Published by Elsevier Science Ltd

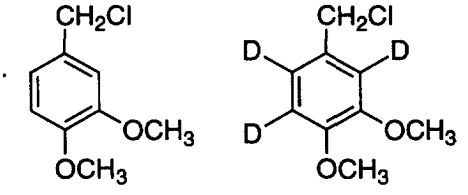
INTRODUCTION

White-rot fungi belonging to the genus *Bjerkandera* are known to produce *de novo* a considerable number of chlorinated aromatic compounds. Chlorinated anisyl metabolites (CAM) are the most common organohalogens produced by these higher fungi [1–4], but the production of chlorinated benzoic acid derivatives has also been reported [5]. From all the *Bjerkandera* species, *Bjerkandera* sp. strain BOS55 is most studied. Up to now, nine chlorinated organic compounds have been analysed in the ethyl acetate extract from the extracellular fluid of the mycelium of this fungus.

During our study on CAM produced by basidiomycetes [6], we have used GC with parallel detection (FID and ECD), prior to GC-mass spectroscopy, to facilitate the detection of halogenated compounds. This combined detection technique revealed the presence of several minor organohalogens with unknown structure which were hard to trace by FID alone. Here we describe one of the minor compounds in the ethyl acetate extracts from *Bjerkandera* sp. BOS55 which is a new organohalogen not previously reported as a *de novo* metabolite from basidiomycetes or any other living organisms.

RESULTS AND DISCUSSION

The fungal strain of *Bjerkandera* sp. BOS55 was cultivated as described [5]. When the culture fluid was completely covered by the mycelium (3 weeks), the culture fluid was filtered and extracted with ethyl acetate following the standard procedure. The resulting sample was then subjected to GC analysis with parallel detection (FID and ECD). Next to the signals of already known halometabolites, a very small FID signal (< 1%) of an unknown compound with high intensity on ECD was observed. The identity of this compound was further investigated with GC-mass spectrometry. Its EI-mass spectrum showed $[M]^+$ and $[M+2]^+$ at *m/z* 186 and 188 in a ratio of 3:1, respectively, suggesting a monochlorinated compound with molecular formula of $C_9H_{11}O_2Cl$. The fragment at *m/z* 151 appeared as the base peak and did not show an isotope peak. Therefore, this ion must be formed by splitting off a Cl radical which is characteristic for benzylic chlorides. These observations and the less intense ion peaks (rel. int.) at *m/z* 173 (0.6), 171 (1.8), 135 (6) and 107 (24) ascribed to $[M+2-Me]^+$, $[M-Me]^+$, $[M-Me-HCl]^+$ and $[M-Me-HCl-CO]^+$, respectively, led to the conclusion that this unknown com-



veratryl chloride veratryl-d_3 chloride

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pound is a dimethoxylated benzylic chloride. Since 3,4-dimethoxybenzyl alcohol is a common metabolite of white-rot fungi [7], the unknown minor halometabolite was tentatively characterized as veratryl chloride (3,4-dimethoxybenzyl chloride). Its identity was unambiguously established through synthesis of an authentic sample of veratryl chloride and comparison of retention times (co-injection) and mass spectral data.

The detection of veratryl chloride in the culture fluid of *Bjerkandera* sp. BOS55 is rather exceptional, because this compound is unstable in aqueous medium as analysis of a solution of veratryl chloride in the culture medium (1.02 mg 10 ml^{-1}) clearly demonstrated. After standing for two weeks at room temperature and standard sample preparation, GC-mass spectral analysis only showed the presence of veratryl alcohol; no trace of veratryl chloride could be observed. After standard workup of a freshly prepared solution of veratryl chloride in the culture medium, GC-mass spectral analysis revealed the presence of small amounts (<5%) of veratryl chloride; the majority of product was veratryl alcohol. Due to the instability of veratryl chloride in aqueous medium, more evidence for its biosynthesis was required. Additional evidence was obtained from experiments in which the stimulation of aryl metabolite production in *Bjerkandera* sp. BOS55 with biosynthetic precursors and lignin degradation products was studied [8]. The addition of benzoic-d_5 acid and 4-hydroxybenzoic-d_4 acid to the culture medium of *Bjerkandera* sp. BOS55 resulted in the formation of deuterated metabolites indicating that these aromatic acids entered into the biosynthetic pathway.

We repeated these experiments with benzoic-d_5 acid and 4-hydroxybenzoic-d_4 acid and, after careful GC-mass spectral analysis of the ethyl acetate extracts, the presence of small but distinct amounts of veratryl-d_3 chloride mixed with some undeuterated veratryl chloride was observed. The EI-mass spectrum of veratryl-d_3 chloride exhibited the $[M+2]^+$ and $[M]^+$ peak at *m/z* 191 (8) and 189 (24), respectively, and a base peak at *m/z* 154. The peaks at *m/z* 191 and 189 indicate that Cl is not attached to the aromatic ring. The appearance of the base peak at *m/z* 154 is only consistent with Cl at the benzylic position. Also the fragmentation pattern was similar to that of veratryl chloride (see Experimental). From these results, it is obvious that veratryl chloride is produced *de novo* by *Bjerkandera* sp. BOS55. The detection of this unstable compound suggests a continuous production by the white-rot fungus. Although the actual mechanism for the formation of veratryl chloride is unclear, it may involve a free-radical halogenation reaction similar to the formation of chloromethyl phenyl ether from anisole [9]. Veratryl chloride is the first natural product of biological origin described which contains a halogenated benzylic carbon.

EXPERIMENTAL

General. Mps: uncorr.; ^1H NMR (200 MHz) and ^{13}C NMR (50 MHz) in $CDCl_3$, δ in ppm (int. standard: TMS). Cultivation of *Bjerkandera* sp. strain BOS55 and sample prepn were performed as described [5]. For the experiments in which benzoic-d_5 acid and 4-hydroxybenzoic-d_4 acid were added to the culture medium of *Bjerkandera* sp. BOS55, see ref. [8]. Samples were subjected to GC with parallel detection and GC-MS.

Detection and identification of halometabolites. GC analyses were performed on a Varian 3600 gas chromatograph equipped with a fused-silica capillary column (DB17, 30 m × 0.25 mm i.d., film thickness: 0.25 μm) and a 1:1 end splitter with each split leading to a separate detector. Parallel detection was carried out by flame ionization (FID) and electron capture (ECD). Carrier gas and flow: N_2 at 1.2 ml min^{-1}. Injector temp. 220°, FID temp. 230°, ECD temp. 275°; temp. programme: 70–250° at 7° min^{-1} hold 20 min. Injection vol.: 10 μl; split ratio 1:100. The FID signals were integrated on the int. integrator of the gas chromatograph. GC-MS analyses were performed on an HP5970B quadrupole mass spectrometer 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°; the temp. programme was identical to that used by GC. Injection vol.: 10 μl split ratio 1:100. EI-MS were obtained at 70 eV. The identification of veratryl chloride and its deuterated form were achieved by comparison of R_t and mass spectra to data of authentic veratryl chloride. All measurements were done in duplicate from a duplicate set of cultures.

Authentic compounds. Veratryl chloride was prepd from veratryl alcohol in 87% yield following the procedure of Adams *et al.* [10]. White solid, mp 51–52° (lit. [11] 50°). ^1H NMR (200 MHz, $CDCl_3$): δ 3.89 and 3.91 (2*s*, 6H, MeO-3 and MeO-4), 4.58 (*s*, 2H, C-α), 6.83 (*d*, J = 8.2 Hz, C-5), 6.93 (*s*, 1H, C-2), 6.97 (*d*, J = 8.2 Hz, C-5). ^{13}C NMR (50 MHz, $CDCl_3$): δ 46.66 (*t*, C-α), 55.88 (2*q*, MeO-3 and MeO-4), 110.95 (*d*, C-5), 111.68 (*d*, C-2), 121.12 (*d*, C-6), 129.97 (*s*, C-1), 149.09 (*s*, C-3 or C-4), 149.19 (*s*, C-3 or C-4).

MS. The spectra of veratryl chloride and veratryl-d_3 chloride are shown below; only relevant peaks with intensities higher than 5% are given.

Veratryl chloride. m/z (rel. int.): 188 $[M+2]^+$ (7), 186 $[M]^+$ (21), 151 (100), 135 (6), 107 (24), 91 (6), 78 (9), 77 (13), 65 (17), 51 (13), 39 (19).

Veratryl-d_3 chloride. m/z (rel. int.): 191 $[M+2]^+$ (8), 189 $[M]^+$ (23), 154 (100), 151 (6)*, 138 (7), 110 (24), 94 (8), 81 (12), 80 (10), 68 (16), 54 (6), 40 (7).

* The ion peak at *m/z* 151 is ascribed to the base peak of undeuterated veratryl chloride.

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