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TRICHLORINATED PHENOLS FROM *HYPHOLOMA ELONGATUM*

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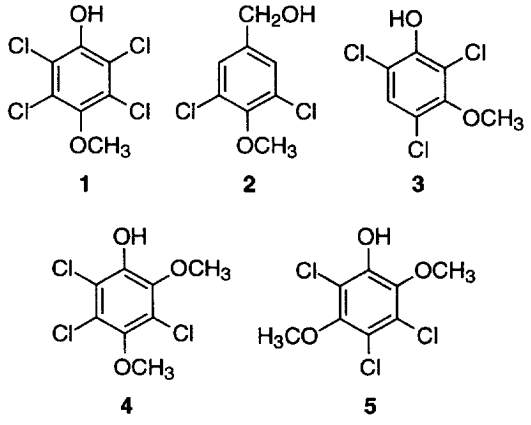
Key Word Index—*Hypholoma elongatum*; Basidiomycetes; novel metabolites; chlorophenols.

Abstract—Three trichlorinated phenols, 2,4,6-trichloro-3-methoxyphenol, 3,5,6-trichloro-2,4-dimethoxyphenol and 3,4,6-trichloro-2,5-dimethoxyphenol, were detected as novel metabolites in the ethyl acetate extract from the culture medium of the Basidiomycete, *Hypholoma elongatum* (strain WIJS94-28). © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Basidiomycetes as decomposers of forest litter represent an ecologically important group of organisms in the environment and are known to produce a wide variety of organohalogens [1]. Three of the most commonly occurring Basidiomycete genera in The Netherlands, *Hypholoma*, *Mycena* and *Bjerkandera*, were found to be dominant among the high producers of organohalogens [2]. Among these organohalogens, chlorinated hydroquinone methyl ethers (CHMs) and chlorinated anisyl metabolites (CAMs) are the most common. The CHM, drosophilin A (tetrachloro-4-methoxyphenol, **1**), was the first halogenated metabolite identified in a Basidiomycete [3] and has a structure very similar to the anthropogenic pentachlorophenol. Apart from drosophilin A, several other CHMs are known from Basidiomycetes. To date, CHMs have been found in 10 different genera [1, 4]. CAM production has so far been reported for 16 Basidiomycete genera [1,5,6]. *Hypholoma elongatum* (strain WIJS94-28), for instance, produces almost selectively 3,5-dichloro-*p*-anisyl alcohol (**2**) in a remarkably high concentration (108 mg l^{-1}), when grown on a medium with a high nitrogen (HN) content [6].

In the present paper, we report the results of a study in which organohalogen production by *H. elongatum* (strain WIJS94-28) grown on a medium with a low nitrogen (LN) content was examined. In contrast to the selective production of 3,5-dichloro-*p*-anisyl alcohol by *H. elongatum* on HN medium [6], five different organohalogens are produced by *H. elongatum* on LN medium. Three of these compounds are novel trichlorinated phenols which were not previously reported as *de novo* metabolites, from Basidiomycetes or any other living organism. The two other organohalogens, drosophilin A and 3,5-dichloro-*p*-anisyl alcohol, are known as *de novo* metabolites from Basidiomycetes. Drosophilin A production by *Hypholoma* has never been demonstrated before.



RESULTS AND DISCUSSION

The fungal strain (*H. elongatum* WIJS94-28) was cultivated in the dark at 25° on LN medium [7]. When the culture fluid was completely covered by mycelium (6 weeks), it was filtered and extracted with ethyl acetate [8]. The identification of chlorinated compounds in the ethyl acetate extract was performed with GC-mass spectrometry, and comparison of retention times and mass spectra to data of respective reference com-

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pounds. The concentration of chlorinated compounds was determined by GC analysis using the internal standard quantitation method [9].

In the ethyl acetate extract, five chlorinated compounds could be detected. Two of these compounds, drosophilin A (**1**) and 3,5-dichloroanisyl alcohol (**2**), produced in concentrations of 0.7 and 40.2 mg l^{-1}, respectively, are known *de novo* metabolites from Basidiomycetes. The production of 3,5-dichloroanisyl alcohol by *Hypholoma* species is well known [5, 6]. On the other hand, the generation of drosophilin A, or any other CHM, by *Hypholoma* has not been reported before.

The identity of the other three chlorinated compounds was unknown. The EI mass spectrum of the first unknown metabolite produced in a concentration of 4.0 mg l^{-1}, showed $[M]^+$, $[M+2]^+$, $[M+4]^+$ and $[M+6]^+$ ions at *m/z* 226, 228, 230 and 232, in a ratio of 10:9.6:3:0.3, respectively, suggesting a trichlorinated compound with molecular formula of $C_7H_5Cl_3O_2$. These data and the prominent ion peaks (rel. int.) at *m/z* 213 (85), 211 (89), 185 (91), 183 (95), 149 (31) and 147 (47), ascribed to $[M+2-Me]^+$, $[M-Me]^+$, $[M+2-Me-CO]^+$, $[M-Me-CO]^+$, $[M+2-Me-CO-HCl]^+$ and $[M-Me-CO-HCl]^+$, respectively, led to the conclusion that this compound is a trichlorinated methoxyphenol [10, 11]. Since GC retention and mass spectral data for all the possible isomers of trichlorinated 2-methoxy- [10] and 4-methoxyphenol [11] clearly differed from those of the unknown metabolite, the methoxyl group in the latter compound is most likely positioned at C-3. In order to gather additional information about the substitution pattern of the unknown compound, some chlorination experiments were performed on 3-methoxyphenol. It was found that treatment of 3-methoxyphenol with molecular chlorine in glacial acetic acid afforded a single product, 2,4,6-trichloro-3-methoxyphenol (**3**), with identical retention time (co-injection) and mass spectral data as the unknown metabolite. The position of the three Cl substituents at C-2, C-4 and C-6 in **3** was deduced from its NMR spectral data [12].

The EI mass spectra of the two other unknown metabolites were similar and both indicated the presence of three Cl atoms. The appearance of $[M]^+$ at *m/z* 256 was consistent with a molecular formula of $C_8H_7Cl_3O_3$ and suggested that these compounds are trichlorinated dimethoxyphenols. In order to establish their identities unequivocally, the six possible isomers of dimethoxyphenol were subjected to exhaustive chlorination. Treatment of 3,5- and 2,6-dimethoxyphenol with molecular chlorine in chloroform afforded 2,4,6-trichloro-3,5-dimethoxyphenol [13] and 3,4,5-trichloro-2,6-dimethoxyphenol [14], respectively, as pure compounds in good yield (ca 70%). Exhaustive chlorination of the other isomers led to rather complex product mixtures in which variable amounts of the corresponding trichlorinated derivatives were detected. All attempts to obtain these trichloro adducts in pure form resulted in demethylation and/or decomposition. Retention times and mass spectral data found for the two pure trichloro adducts did not match with those for the unknown trichlorinated dimethoxyphenols produced by *H. elongatum*. Careful GC-mass spectral analysis of the four crude product mixtures prior to work-up, however, revealed that the trichlorinated products derived from 2,4- [15] and 2,5-dimethoxyphenol [16], i.e. 3,5,6-trichloro-2,4-dimethoxyphenol (**4**) and 3,4,6-trichloro-2,5-dimethoxyphenol (**5**), respectively, were identical with the two remaining unknown metabolites. Since pure reference compounds were not available, only the relative concentration of **4** and **5** (1.5 and 2.6%, respectively) in the extract could be determined by means of GC-MS analysis. 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. Previously, it was reported that 3,5,6-trichloro-2,4-dimethoxyphenol (**4**) might be a degradation product of tetrachloroguaiacol by *Rhodococcus chlorophenolicus* [17, 18].

Hypholoma elongatum is an ecologically important Basidiomycete inhabiting moss (*Spaghnum* and *Poytrichum*) in wetlands. The results presented here indicate that this fungus might be an important source of naturally produced chlorophenols in ecosystems. Chlorophenols are susceptible to polymerization reactions by phenol oxidizing enzymes [19], which might account for the unusually high organohalogen content of humus recovered from wetlands [20].

EXPERIMENTAL

General

Mps: uncorr. ^1H NMR (200 MHz) and ^{13}C NMR (50 MHz) $CDCl_3$ with TMS as int. standard.

Organism and culture conditions

Hypholoma elongatum (strain WIJS94-28) was obtained from the Culture Collection of Industrial Microbiology (CIMW), Wageningen Agricultural University, The Netherlands. The fungal strain was grown on LN medium [7] with the addition of NaCl (0.06 g). Medium (10 ml) 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 fresh 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 (6 weeks), the culture fluid was harvested.

Extraction and sample preparation

Filtration and extraction of the culture fluid were performed as described in Ref. [8]. After removal of EtOAc under red. pres., the resulting residue was redissolved in 0.25 ml of freshly dist. EtOAc con-

taining 54.5 μg of 4-bromoanisole as int. standard, and then subjected to GC-MS analysis.

Identification and quantitation of chlorometabolites

Samples were analysed on a quadrupole MS coupled to a GC equipped with a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d., film thickness: 0.25 μm). Carrier gas and flow: He at 1.0 ml min^{-1}. Injector temp. 220°; temp. programme: 70–250° at 7° min^{-1}, hold 25 min. Injection vol.: 5.0 μl; split ratio 1:5. EIMS were obtained at 70 eV. Identification of chlorinated compounds was achieved by comparison of R_ts and MS to data of respective authentic compounds. The concns of **1**–**3** were determined by GC analysis using the int. standard quantitation method [9]. Because authentic compounds **4** and **5** could not be synthesized in pure form (see below), quantitation of these compounds was not possible. Based on the total ion current, an indication of the rel. concn of **4** and **5** in the extract was obtained by GC-MS. All measurements were done in duplicate from a duplicate set of cultures.

Authentic compounds

Drosophilin A (**1**) was kindly provided by Dr J. Knuutinen (University of Jyväskylä, Finland). 3,5-Dichloro-*p*-anisyl alcohol (**2**) was prepd as described in Ref. [5].

Preparation of 2,4,6-*trichloro*-3-*methoxyphenol* (**3**). A soln of 3-methoxyphenol (2.5 g, 20.2 mmol) in HOAc (12.5 ml) was purged with Cl_2 at room temp. for 30 min. The reaction mixt. was poured into H_2O (100 ml) and extracted with EtOAc (3 × 50 ml). The combined organic layers were washed with H_2O, satd aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concd *in vacuo*. Kugelrohr short-path distillation (110° at 10^{-2} mbar) of the remaining residue gave a yellow oil (4.3 g) which was purified by CC on silica gel (petrol–EtOAc, 20:1). Yield: 3.3 g (72%) **3**, yellow crystals, mp 62–66° (lit. [21] 64°). ^1H NMR (200 MHz, $CDCl_3$): δ 3.88 (*s*, 3H, MeO-3), 5.90 (*s*, 1H, HO-1) 7.30 (*s*, 1H, H-5). ^{13}C NMR (50 MHz, $CDCl_3$): δ 60.87 (*q*, MeO-3), 116.25 (*s*, C-2 or C-6), 116.94 (*s*, C-2 or C-6), 120.11 (*s*, C-4), 127.89 (*d*, C-5), 147.69 (*s*, C-1), 151.84 (*s*, C-3). EIMS *m/z* (rel. int.): 232 (3) $[M+6]^+$, 230 (30) $[M+4]^+$, 228 (95) $[M+2]^+$, 226 (100) $[M]^+$, 215 (28), 213 (85), 211 (89), 187 (29), 185 (91), 183 (95), 149 (31), 147 (47), 121 (26), 119 (42), 84 (22); HRMS: $[M]^+$ *m/z* 225.9349 (calcd for $C_7H_5Cl_3O_2$, 225.9355).

Preparation of 3,5,6-*trichloro*-2,4-*dimethoxy phenol* (**4**). 2,4-Dimethoxyphenol was prepd as described in Ref. [15]. A soln of 2,4-dimethoxyphenol (15 mg, 0.1 mmol) in $CHCl_3$ (2 ml) was purged with Cl_2 at room temp. for 2 min. GC-MS analysis of the crude reaction mixt. revealed the presence of **4** (ca 5%). EIMS *m/z* (rel. int.): 260 (26) $[M+4]^+$, 258 (78) $[M+2]^+$, 256 (74) $[M]^+$, 245 (27), 243 (94), 241 (100), 215 (33), 213 (35), 200 (43), 198 (49), 103 (29), 87 (44); HRMS: $[M]^+$ *m/z* 255.9463 (calcd for $C_8H_7Cl_3O_3$, 255.9461). All attempts to obtain pure **4** from the crude reaction mixt. failed. Other chlorinating agents did not produce **4**.

Preparation of 3,4,6-*trichloro*-2,5-*dimethoxyphenol* (**5**). 2,5-Dimethoxyphenol was prepd as described in Ref. [16]. A soln of 2,5-dimethoxyphenol (0.25 g, 1.62 mmol) in CH_2Cl_2 (15 ml) was treated with excess benzyltrimethylammonium tetrachloroiodate [22]. Work-up and purification by CC on silica gel (petrol–EtOAc, 4:1) gave 0.22 g (61%) of the corresponding dichloro compound. To a soln of this compound in HOAc (15 ml) was added dropwise sulfuryl chloride (80 μl) and the reaction mixt. stirred at room temp. for 3 h. According to GC-MS analysis, the crude reaction mixt. contained ca 65% of **5**. EIMS *m/z* (rel int.): 260 (16) $[M+4]^+$, 258 (43) $[M+2]^+$, 256 (52) $[M]^+$, 245 (32), 243 (91), 241 (100), 215 (31), 213 (31), 200 (36), 198 (36), 103 (37), 87 (45); HRMS: $[M]^+$ *m/z* 255.9477 (calcd for $C_8H_7Cl_3O_3$, 255.9461). Attempts to obtain pure **5** from the crude reaction mixt. by CC failed due to demethylation and decomposition.

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