

Organohalogen Metabolites of Basidiomycetes

Abstract

The biological capacity for organohalogen synthesis is widespread among basidiomycetes. Up to 79 organohalogen metabolites have been identified from 46 genera of basidiomycetes. Three of the most common occurring basidiomycete genera in The Netherlands were found to be responsible for the highest levels of organohalogen production. Based on the biodiversity of species producing chlorinated anisyl metabolites (CAM), they represent the most ubiquitous group of organohalogens from basidiomycetes. Phenylalanine and 3-chloro-4-hydroxybenzoate were implicated as possible precursor of CAM biosynthesis. Organohalogens were shown to be significantly produced when basidiomycetes were cultured on natural lignocellulosic substrates. Likewise, CAM could be detected in the environment and were highly concentrated in rotting wood and forest litter colonized by common fungi. Considering the ecological importance of basidiomycetes as decomposers of forest litter, we conclude that they are a major source of natural organohalogens in terrestrial environments.

Introduction

Basidiomycetes are higher fungi that produce macroscopic fruiting bodies (mushrooms). These organisms play a key role in the ecology degrading lignocellulosic debris such as wood, straw and litter. Also basidiomycetes are known to constitute a major fraction of the living biomass degrading forest litter. Approximately a ton dry weight of basidiomycete mycelium is estimated to be produced annually per hectare in a typical temperate hardwood forest (Swift, 1982). Over the years, several basidiomycetes were reported to produce *de novo* organohalogen metabolites indicating that this group of organisms might be an important source of naturally occurring organohalogens in terrestrial environments (De Jong *et al.*, 1994a). Additionally, organohalogen production has been shown to be associated with the decay of forest litter (Oberg *et al.*, 1996a). The objective of this manuscript is to review the literature and report on our ongoing

studies demonstrating the widespread capacity among basidiomycetes to produce organohalogens in the environment.

Organohalogen screening

Two studies have been conducted screening basidiomycetes for bulk organohalogen parameters. Either the production of adsorbable organic halogens (AOX) or total organic halogens (TOX) were measured (Verhagen *et al.*, 1996; Oberg *et al.*, 1997). The combined results of both studies screened 200 strains and evidence was found for organohalogen production in 51% of the strains, indicating a widespread capacity for organohalogen biosynthesis among basidiomycetes. Approximately 10% of all strains produced organohalogens at remarkably high concentrations, ranging from 5 to 67 mg AOX/l in the extracellular culture fluid. The top AOX producers were predominated by species from three genera: *Bjerkandera*, *Mycena* and *Hypholoma* (Verhagen *et al.*, 1996). These genera are reported to be the 6th, 2nd and 1st most common occurring basidiomycetes in The Netherlands; respectively (Nauta and Vellinga, 1995).

Organohalogen metabolites

In 1995, we reviewed the literature indicating that 53 halogenated metabolites have been reported as de novo products of basidiomycetes (Field *et al.*, 1995). Since then, there have been new reports and newly found reports published prior to 1995 which brings the total to 79 halometabolites identified from 46 genera of basidiomycetes. Compared with the data from the AOX and TOX screenings, an additional 21 genera are known to produce organohalogens, although no metabolites have yet been identified.

The organohalogen metabolites of basidiomycetes can be classified as halomethanes, miscellaneous halogenated aliphatics and halogenated aromatics (Field *et al.*, 1995). Six halomethane metabolites are known from 11 genera, ranging from chloromethane to dichloriodomethane. Up to 8 other chlorinated aliphatics metabolites have been described from 4 genera: pinicologlyoxal from *Resinicium pinicola*; lepiochlorin from *Lepiota* sp.; 1-chloro-heptadecyne from *Lactarius* sp. and 5 different chlorinated pentenoic or hexenoic amino acids from the genus *Amanita* (references in Field *et al.*, 1995; Anonymous, 1993; Ohta *et al.*, 1987; Hatanaka *et al.*, 1994). As many as 65 halogenated aromatic compounds have been identified from 34 genera of basidiomycetes. Common occurring chlorinated aromatic metabolites of basidiomycetes are illustrated in Figure 1 and the genera producing these are listed in Table 1. The most ubiquitous of these are the chlorinated anisyl metabolites (CAM) which represent 9 metabolites that are known from 16 genera. CAM are produced at relatively high concentrations (ranging from 10 up to 108 mg/l) in many strains including

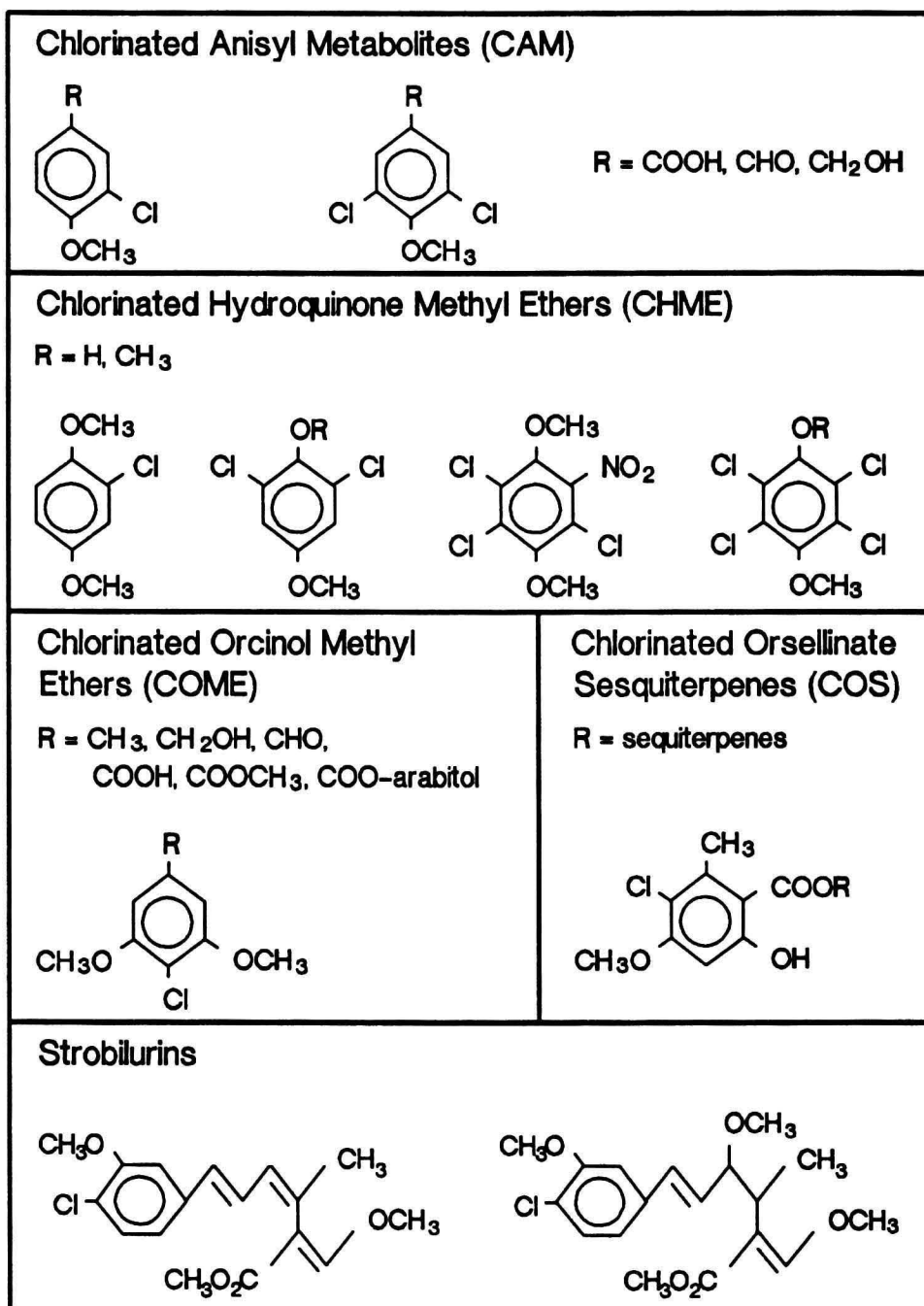


Fig. 1. Structures of common chlorinated aromatic metabolites produced de novo by basidiomycetes (literature references in Table 1).

Table 1. Basidiomycete genera producing ubiquitous chlorinated aromatic metabolites.

Metabolite Family*	Genera	Reference***
CAM	<i>Bjerkandera</i>	1, 2
	<i>Daedaleopsis</i> (t)**	3
	<i>Formes</i> (t)	3
	<i>Hypholoma</i>	1, 2
	<i>Ischnoderma</i> (t)	3
	<i>Lepsita</i>	1, 4
	<i>Mycena</i>	2
	<i>Oudemansiella</i>	1
	<i>eniophora</i>	2
	<i>Phellinus</i>	2
	<i>Pholiota</i>	1, 2
	<i>Phylloporia</i>	2
	<i>Pleurotus</i>	1
	<i>Ramaria</i>	3
	<i>Stropharia</i>	1, 5
	<i>Trametes</i>	
CHME	<i>Agaricus</i>	1, 6
	<i>Bjerkandera</i>	1, 6
	<i>Coprinus</i>	1
	<i>Hypholoma</i> (t)	7
	<i>Lepista</i>	4
	<i>Mycena</i>	1
	<i>Peniophora</i>	6
	<i>Phellinus</i>	1, 6
	<i>Psathyrella</i>	1
	<i>Russula</i>	1
	<i>Schizophyllum</i>	8
COME	<i>Bjerkandera</i>	6
	<i>Hericium</i>	1, 9
COS	<i>Armillaria</i>	1, 10, 11
	<i>Clitocybe</i>	12
Strobilurins	<i>Mycena</i>	13
	<i>Strobilurus</i>	13
	<i>Xerula</i>	14

* abbreviations defined in Figure 1;

** trace levels of metabolite

*** 1) references cited in Field *et al.*, 1995; 2) Swarts *et al.*, 1996a; 3) De Jong *et al.*, 1994a; 4) Hjelm *et al.*, 1996; 5) Thines *et al.*, 1995; 6) Teunissen *et al.*, submitted; 7) Verhagen *et al.*, unpublished data; 8) Schwartz *et al.*, 1992; 9) Qian *et al.*, 1990; 10) Arnone *et al.*, 1988a; 11) Cremin *et al.*, 1995; 12) Arnone *et al.*, 1988b; 13) references cited in Anke, 1995; 14) Anke *et al.*, 1983

ecologically significant species. The second most important group are the 6 different chlorinated hydroquinone methyl ethers (CHME) produced by 11 genera. Furthermore, two genera of basidiomycetes produce 6 different types of chlorinated orcinol methyl ethers (COME) and another two genera produce 10 different metabolites of the structurally related chlorinated orsellinate sesquiterpenes (COS). Finally, three genera produce two types of chlorinated strobilurin metabolites.

New chlorinated aromatic metabolites identified since our last review are shown in Figure 2 (see references in figure caption). Five new metabolites were described from strains in the genus *Bjerkandera*, these included 3-chloro- or 3,5-dichloro-4-hydroxybenzoates as well as 2,4-dichlorobenzoate. 5-chloroveratraldehyde was identified from *Lepista nuda*. Two types of chlorinated 3-(4-methoxyphenyl)propanoid structures, methyl chloro-4-methoxycinnamate and trametol were identified from *Poria cinerescens* and *Trametes* sp; respectively. In addition to these, a polychlorinated tetrameric metabolite, denominated russuphelol, was described from the black pigments in *Russula subnigricans* fruiting bodies and is presumably formed from the oxidative coupling of 2,6-dichloro-4-methoxyphenol (Ohta *et al.*, 1995).

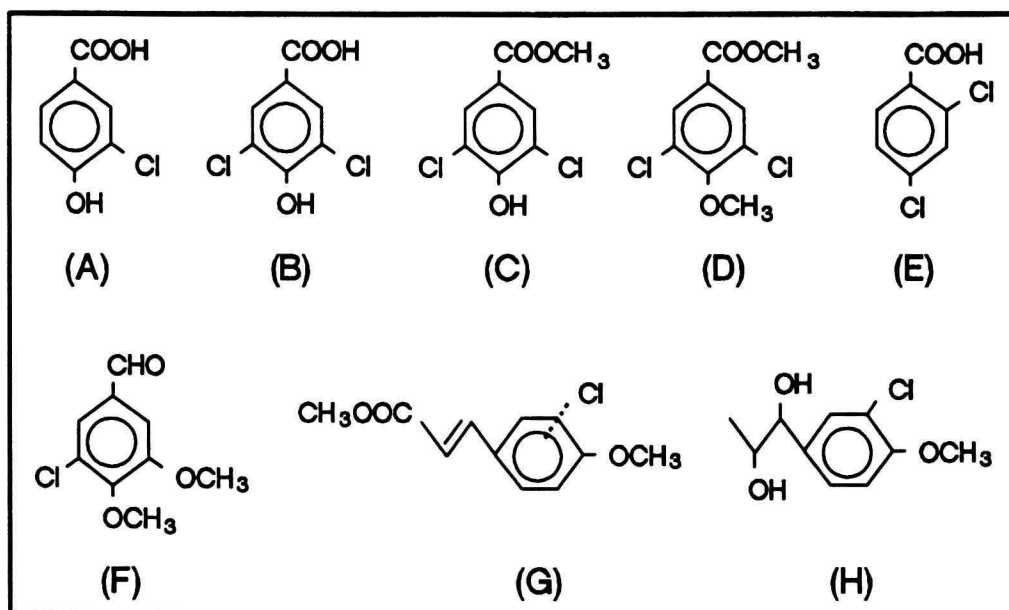


Fig. 2. New aromatic metabolites of basidiomycetes published in 1995 and 1996. (A) 3-chloro-4-hydroxybenzoate; (B) 3,5-dichloro-4-hydroxybenzoate; (C) methyl 3,5-dichloro-4-hydroxybenzoate and (D) methyl 3,5-dichloro-4-anisate reported from *Bjerkandera* spp. by Swarts *et al.* (1996b). (E) 2,4-dichlorobenzoate reported from *Bjerkandera adusta* by Öberg *et al.* (1996b). (F) 5-chloroveratraldehyde reported from *Lepista nuda* by Hjelm *et al.* (1996). (G) methyl chloro-4-methoxycinnamate reported from *Poria cinerescens* by Öberg *et al.* (1996b). (H) trametol reported from *Trametes* sp. by Brambilla *et al.* (1995).

Biosynthesis

Not much is known about the biosynthesis of organohalogenes by basidiomycetes. In the case of halomethanes, the formation of chloro-, bromo- and iodomethane probably proceeds via methylation of an inorganic halide by a S-adenosylmethionine dependent methyl transferase as has been shown to be the case for algae and plants (Wuosmaa and Hager, 1990; Attieh *et al.*, 1995). In *Amanita* spp. the chlorinated pentenoic and hexenoic amino acids probably arise via biological chlorohydrin reactions of penta- or hexadienoic amino acids which are also present (Ohta *et al.*, 1987; Hatanaka *et al.*, 1994). CAM and chlorinated 4-hydroxybenzoate metabolites would likely result from the action of chloroperoxidase since the chlorination pattern is almost exclusively ortho to the hydroxy (or methoxy) groups (Franssen and van der Plas, 1992). However, to date chloroperoxidases have not yet been isolated from basidiomycetes. The halogenating system appears to have a high affinity for chloride since increasing chloride concentrations beyond background levels in chloride limited culture medium or wood substrates did not improve organohalogen production (De Jong *et al.*, 1994a; Verhagen *et al.*, 1996). Also a preference for bromide is indicated by the formation of bromoanisyl metabolites instead of CAM in media supplemented with KBr (Spinnler *et al.*, 1994).

Evidence is accumulating that CAM are synthesized via the phenylpropanoid metabolism of phenylalanine. The addition of phenylalanine to cultures of *Bjerkandera* sp. resulted in 10-fold increases in CAM production (Mester *et al.*, 1997). Benzoate is known to be an intermediate in the biosynthesis of veratryl alcohol (3,4-dimethoxybenzyl alcohol) from phenylalanine (Jensen Jr. *et al.*, 1994). Consequently, deuterated benzoate and deuterated 4-hydroxy- and 3-chloro-4-hydroxybenzoates were tested as precursors to CAM biosynthesis. Addition of any of these compounds to the *Bjerkandera* cultures resulted in an incorporation of the deuterated label into CAM metabolites as evidenced by matching retention times and mass spectrum in the GC-MS with the exception of the difference in mass between deuterium and hydrogen (Mester *et al.*, submitted). These results combined with the fact 3-chloro-4-hydroxybenzoate is known as a de novo metabolite from *Bjerkandera* strains (Figure 2) implicate this compound as a likely intermediate in the biosynthesis of CAM. Chlorination of 4-hydroxybenzoate was demonstrated indicating that 4-hydroxybenzyl compounds are possible substrates of the halogenation system. Conversion of 3-chloro-4-hydroxybenzoate to CAM would be expected to proceed via methylation of the 4-hydroxy group and reduction of the carboxylic acid group. Both S-adenosylmethionine dependent methyl transferases and a chloromethane dependent methylating systems are known from basidiomycetes (Coulter *et al.*, 1993; De Jong *et al.*, 1994b). Aryl dehydrogenases are also common intracellular enzymes in basidiomycetes, including *Bjerkandera* (De Jong *et al.*, 1994b). However, chlorination and methylation can also occur at an earlier stage of the phenylpropanoid metabolism as is suggested by the occurrence of trametol and methyl chloro-4-methoxycinnamate as metabolites from other basidiomycetes (Figure 2).

Organohalogens from basidiomycetes in the environment

The occurrence of organohalogen metabolites in the environment is inferred from the ability of various basidiomycete strains to produce AOX or TOX when cultured on natural lignocellulosic substrates in the laboratory (Verhagen *et al.*, 1996; Oberg *et al.*, 1997). Figure 3, illustrates the extent of this production after culturing *Hypholoma fasciculare* on different natural substrates for 6 weeks. Up to 132 mg AOX/kg dry weight of forest litter substrate was produced. *Mycena metata* produced up to 193 mg AOX/kg of forest litter substrate (Verhagen *et al.*, 1996). The maximum specific rates of organohalogen production for these species on natural substrates is extremely high. Values ranging from 630 to 3200 mg AOX produced per kg of mycelium dry weight per day were found with *Hypholoma fasciculare* when colonizing wood and forest litter utilizing ergosterol measurements to estimate the mycelium biomass (Verhagen *et al.*, unpublished data). Similar specific production rates were observed by direct measurement of the biomass in defined liquid medium. The maximum rates of organohalogen production occurred during the transition of the fungal culture from primary to secondary metabolism.

Likewise, specific compounds such as CAM are also produced by basidiomycetes when cultured on wood and straw (De Jong *et al.*, 1994a; Oberg *et al.*, 1997). However, the real proof that such metabolites exist in the environment is to look at their concentrations in environmental samples. Environmental samples were collected from rotting wood and decomposed forest litter colonized by basidiomycetes. The colonies of basidiomycetes were localized by their fruiting bodies (De Jong *et al.*, 1994a; Hjelm *et al.*, 1996). These samples contained CAM compounds at concentrations ranging from 10 to 70 mg/kg dry weight for various common species from the genera, *Bjerkandera*, *Hypholoma*, *Stropharia*, *Lepista* and *Pholiota*. CAM compounds were also identified at many sites in composite samples of the forest litter (De Jong *et al.*, 1994a).

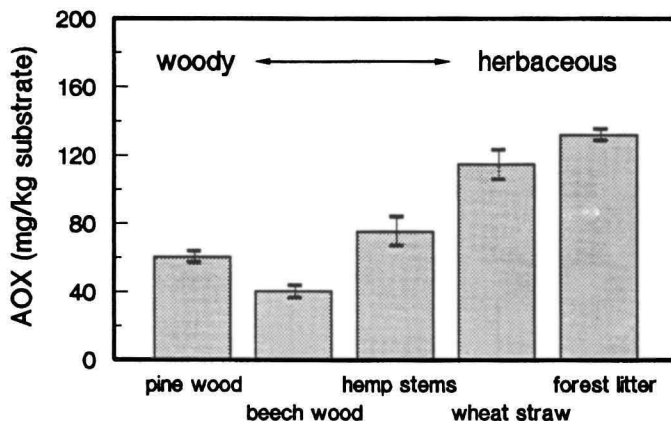


Fig. 3. The net production of adsorbable organic halogens (AOX) by *Hypholoma fasciculare* cultured for 6 weeks on various natural substrates (Verhagen *et al.*, 1996).

Conclusions

A vast number of basidiomycetes were shown to be capable of organohalogen biosynthesis. Significant production of organohalogens by basidiomycetes in the environment was demonstrated by culturing selected strains on natural substrates. Likewise, the common occurring chlorinated anisyl metabolites were found at high concentrations in environmental samples of rotting wood and forest litter. These results combined with the dominant ecological role of basidiomycetes as decomposers of forest litter suggest that they are a major source of natural organohalogens in terrestrial environments.

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