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# NEW HALOMETABOLITES FROM *CALDARIOMYCES FUMAGO*

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**Key Word Index**—*Caldariomyces fumago*, Deuteromycetes, halometabolites, halogenated compounds, dichloroethanol, chloropropanols, chlorohydroxyphenylethanols

**Abstract**—The culture medium of the mould *Caldariomyces fumago* was extracted with three organic solvents and the extracts were analysed by GC-MS Twelve halogen-containing metabolites were found in the dichloromethane extract Seven of these compounds were sufficiently abundant to allow the identification of the new halometabolites, 2,2-dichloroethanol, 1,3-dichloropropanol-2, 1,1,3-trichloropropanol-2, 1,1,3,3-tetrachloropropanol-2, 2-(3-chloro-4-hydroxyphenyl)ethanol and 2-(3,5-dichloro-4-hydroxyphenyl)ethanol in addition to the well-known caldariomycin (2,2-dichloro-1*S*,3*S*-dihydroxycyclopentane). Their possible biosynthetic routes and the implications for the reaction mechanism of the *Caldariomyces* halogenating enzyme chloroperoxidase are discussed

## INTRODUCTION

The occurrence of haloorganic compounds in living matter is a rapidly growing field of interest in natural product chemistry Over 700 halometabolites are known to date The subject has been reviewed in general [1] as well as for individual classes of organisms [2–7]. Some of the authors concerned also briefly discuss the biosynthesis of these compounds [2, 4–7] The role of the enzymes responsible for the actual halogenation step has, until recently [8], only been mentioned without further comment. This is remarkable, since the discovery of the chloroantibiotic caldariomycin (2,2-dichloro-1*S*,3*S*-dihydroxycyclopentane) in the culture medium of the mould *Caldariomyces fumago* [9] directly induced the search for and the detection of the halogenating enzyme chloroperoxidase [10, 11], now one of the most studied halogenating biocatalysts. This very versatile enzyme has been used in several laboratories for the synthesis of chlorinated or brominated steroids [12], alkenes [13, 14], cyclopropanes [15] as well as chlorinated or brominated derivatives of the heterocycles thiazole [16], antipyrine [17] and barbituric acid [18–21] In these investigations as well as in those from other laboratories [22, 23] conflicting results were presented regarding the reaction mechanism of the enzyme; in particular the stereospecificity of the halogenation reaction was a matter of debate.

Some of the controversy may have been caused by the fact that only a minority of the researchers used cyclopentanedione, the only known natural substrate of the enzyme It would be interesting to know whether there are other natural substrates for chloroperoxidase, and whether this enzyme could halogenate them in a stereo- or regiospecific manner. Therefore, we wanted to have a more detailed picture of the natural products, formed in the culture medium of *C. fumago*.

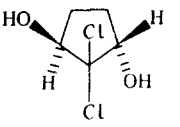
When Clutterbuck *et al.* [9] extracted the culture medium of *C. fumago* with ethyl acetate (EtOAc), they not only isolated white crystals of caldariomycin, but also noted that "an oil remained finally from which nothing crystalline has been separated, but which contains considerable amounts of Cl in organic combination" This induced us to re-examine the culture medium using GC-MS. Halogen-containing compounds are readily recognized by this technique because of the characteristic $^{35}Cl/^{37}Cl$ and $^{79}Br/^{81}Br$ isotope patterns

In this paper we report on the extraction and characterization by GC-MS of six new chloro-containing metabolites from the culture medium of *C. fumago*. The biosynthesis of these compounds and their implications for the reaction mechanism and possible stereospecificity of chloroperoxidase are discussed.

## RESULTS AND DISCUSSION

*Caldariomyces fumago* was grown as described in the Experimental and the filtered culture medium was extracted successively with petrol 60–80°, $CH_2Cl_2$ and EtOAc, in order to separate the polar from the less polar compounds. The extracts were analysed by GC-MS The petrol fraction was practically devoid of any compounds and was not analysed further. Gas chromatography of the EtOAc fraction and the EtOAc continuous extract showed 2,3-butanediol, caldariomycin (**1**), 2-(4-hydroxyphenyl)ethanol (**9**) and an unhalogenated compound of unknown structure* as major constituents. We concentrated our efforts on the dichloromethane ($CH_2Cl_2$) extract because of the large number of compounds present therein The $CH_2Cl_2$ extract appeared to contain a considerable number of small, relatively simple unhalogenated molecules as well as 12 chlorinated organic compounds, one of which was caldariomycin (**1**) Five of the 11 new chlorometabolites of *C. fumago* were only present in very small amounts and could not be

*The mass spectrum of this compound contains the following peaks. *m/z* (rel. int.): 27 (6), 29 (6), 31 (12), 39 (16), 41 (31), 51 (7), 53 (11), 55 (9), 65 (6), 69 (25), 81 (7), 83 (6), 97 (100), 98 (7), 109 (12), 110 (6), 111 (15), 128 (52)



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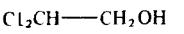

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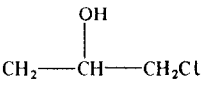

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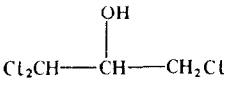

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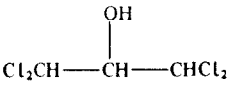

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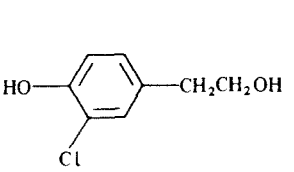

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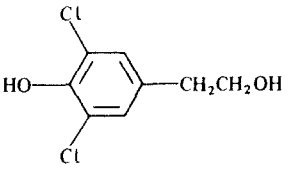

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analysed further. Six compounds, however, gave good mass spectra and comparison with literature data and authentic samples allowed their complete identification

The six new halometabolites could be divided into two groups, chloroethanols/propanols and chlorohydroxyphenylethanols (Table 1) The first group consists of 2,2-dichloroethanol (**2**), 1,3-dichloropropanol-2 (**3**), 1,1,3-trichloropropanol-2 (**4**) and 1,1,3,3-tetrachloropropanol-2 (**5**) The structure of **2** was assigned by comparison of its mass spectrum with literature data [24] The structure of **3** was established by comparison of its mass spectrum with authentic material, further support was obtained by the fact that **3** in the $CH_2Cl_2$ extract coeluted with authentic **3** on GC-analysis The fragmentation patterns of **3**–**5** are very similar the $[M]^+$ peak is not visible, but the spectra show characteristic peaks at *m/z* 79 and 113, corresponding to the α-cleavage products These fragments subsequently lose HCl (peaks at *m/z* 43 and 77, respectively). Compounds **3**–**5** also showed a good correlation between their $R_t$s and their $M_r$, as was found earlier for a series of halogenated isopropanols [25] The mass spectrum of **5** is in good agreement with that published previously [25]

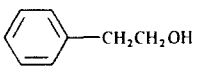
Table 1 Halometabolites and related compounds found in the $CH_2Cl_2$ extract of the culture medium of *Caldariomyces fumago*

| Total ion current (arbitrary units)* | Compound no | Compound |
|---|---|---|
| 539 | **2** | 2,2-Dichloroethanol |
| 1226 | **3** | 1,3-Dichloropropanol-2 |
| 2827 | **4** | 1,1,3-Trichloropropanol-2 |
| 9695 | **8** | 2-phenylethanol |
| 3061 | **5** | 1,1,3,3-Tetrachloropropanol-2 |
| 1313 | **1** | Caldariomycin (2,2-dichloro-1*S*-3*S*-dihydroxycyclopentane) |
| 3111 | | phenylacetic acid |
| 1285 | **6** | 2-(3-Chloro-4-hydroxyphenyl)ethanol |
| 1094 | **9** | 2-(4-Hydroxyphenyl)ethanol |
| 4686 | **7** | 2-(3,5-Dichloro-4-hydroxyphenyl)ethanol |

*Indication of the relative concentration of the compound in the extract

The second group of halometabolites consists of 2-(3-chloro-4-hydroxyphenyl)ethanol (**6**) and 2-(3,5-dichloro-4-hydroxyphenyl)ethanol (**7**) Their structures were confirmed by comparing their $R_t$ and mass spectra with those of authentic samples prepared by reaction of 2-(4-hydroxyphenyl)ethanol with HOCl These chlorometabolites have not been described previously However, some analogues are known. 2-Phenylethanol (**8**, phenetol) and 2-(4-hydroxyphenyl)ethanol (**9**, tyrosol) are well-known compounds occurring in many moulds, and *Marasmius palmivorus* produces 3-chloro-4-hydroxyphenylacetic acid [5] These metabolites are derived from the shikimic acid route, as is caldariomycin [26]. Compounds **6** and **7** are probably synthesized via the shikimic acid pathway, but another route cannot be ruled out. For instance, *Candida* sp. and various pathogenic fungi produce (**9**) as the decomposition product of tyrosine [5]. We found large amounts of tyrosol in the culture medium of *C. fumago* (Table 1), so this may well be a precursor for the chlorinated tyrosols **6** and **7** To test this hypothesis we incubated tyrosol with the pure *Caldariomyces* halogenating enzyme, chloroperoxidase, and found that it was readily converted to 2-(3-chloro-4-hydroxyphenyl)ethanol (**6**) Remarkably, 2-(3,5-dichloro-4-hydroxyphenyl)ethanol (**7**) was formed in only small amounts, even after prolonged incubation.

Since it is well-known that tyrosine is a substrate for chloroperoxidase [27], four possibilities for the formation of the chlorine–carbon bonds ultimately leading to **6** and **7** can be advanced· (i) chlorination of tyrosine residues in proteins [28], (ii) chlorination of free tyrosine, related or not to protein synthesis or degradation, (iii) chlorination of tyrosol and (iv) chlorination of inter-



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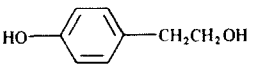

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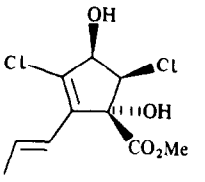
mediates in the shikimic acid route, as found for caldariomycin (**1**). Since **6** can be formed from tyrosol by chloroperoxidase, as already noted above, all four possible biosynthesis routes may be valid for **6**. However in the case of **7** route (iii) is unlikely because **6** proved to be a poor substrate for the enzyme. More information is needed before the biosynthesis of **6** and **7** is known with certainty.

The origin of the dichloroethanol and chloropropanols (**2–5**) is even more intriguing. Compounds of this type, are only known from marine organisms. 1,3-Dichloropropanol-2 (**3**) has been found in Antartic krill [29], and Woolard *et al.* [25] detected a large number of haloacetones and haloisopropanols in the $CH_2Cl_2$ extract of vacuum-dried *Asparagopsis taxiformis* (Rhodophyta, Bonnemaisoniaceae), including our compound **5**. The halometabolites **2** and **4** have not been found before The above authors did not study the biosynthesis of these compounds, but since neither ethanol nor acetaldehyde, propanol-2 or acetone are substrates for chloroperoxidase, we feel that metabolites **2–5** must be derived from larger molecules. Perhaps these larger precursors are molecules like compound **10**, from *Periconia macrospinosa*, and cryptosporiopsin (**11**, from *Phialophora asteris*, *Octospora carbonigena* and many *Cryptosporiopsis* species). These mould metabolites contain the 1,3-dichloropropanol-2 and 1,3-dichloroacetone moiety, respectively, and possibly these compounds are the precursors of the compounds **2–5** or the corresponding chloroacetone derivatives* The hypothesis described above is speculative especially since it does not explain the occurrence of tri- and tetrachloroisopropanols

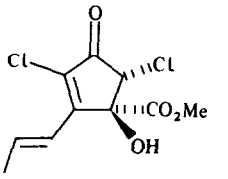
The biological function of the halometabolites is unclear so far but there is some evidence that these compounds play a role in the defence mechanism of the organisms [8]. It is interesting to mention that chloroisopropanols are bactericides [31] and that 2-phenylethanol (**8**), the major constituent of the $CH_2Cl_2$-extract of *C. fumago*, shows antimicrobial activity [32, 33].

One or more halogenation steps are involved somewhere in the biosynthesis route for the formation of **1–7**. The enzyme responsible for these conversions is chloroperoxidase, an extracellular enzyme produced in relatively large amounts by *C. fumago*. Whether this enzyme can act in a stereospecific manner is still not established [19, 34–36]

Studying the stereochemistry of the natural substrates and products of the enzyme possibly can throw light on this matter Five of the halometabolites found here are achiral, **4** has a chiral central C-atom, but since it was not possible to isolate this compound its stereochemistry could not be studied. The key step in the biosynthesis of caldariomycin (**1**) is the conversion of cyclopentane-1,3-dione into its 2,2-dichloro derivative, again a reaction with no need for stereochemical control We conclude from the structures of the compounds found in this study that chloroperoxidase has no need to be a stereospecific chlorinating agent. One should, however, bear in mind that the growth medium of *C myces* probably contains besides the small and volatile halometabolites larger non-volatile halogenated compounds which cannot be detected by GC-MS. Until these molecules are fully characterized we cannot conclude that chloroperoxidase can catalyse halogenation reactions in a stereospecifical manner.




**10**




**11**

*Brewer's yeast can convert 1,1-dichloroacetone into 1,1-dichloropropanol-2 [30]

## EXPERIMENTAL

*General* Chloroperoxidase (CPO) was isolated as described previously [37] Extraction solvents were dist prior to use.

*Growth of mould and isolation of metabolites C fumago* Woron strain CBS 123 26 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands The mould was grown on Czapek-Dox medium, consisting of 50 g of glucose, 2 g of $NaNO_3$, 1 g of $KH_2PO_4$, 0 5 g of KCl, 0 5 g of $MgSO_4$ $7H_2O$, 0.02 g of $FeSO_4$ $7H_2O$ and 3 3 g of yeast extract per l of deionized $H_2O$ The mould was grown for 7 days in a 1 l flask and then transferred to a 20 l flask where it was grown for 9–10 days. Subsequently the cells were centrifuged and the cell-free supernatant concd in an Amicon hollow-fibre apparatus with an $H_{10}P_{10}$-20 cartrige ($M_r$ cutoff = 10 000). The 4 5 l of clear culture medium thus obtained was divided into two equal portions and each portion was extracted with 3 × 500 ml of petrol 60–80°, $CH_2Cl_2$ and EtOAc, successively The residue was then extracted continuously with EtOAc for 24 hr The extracts were dried ($MgSO_4$), concd with a rotary evaporator at 30° and analysed

*GC-MS analysis* The extract (2 μl) was injected into a GC equipped with a SIL19 WCOT capillary column, 26 m × 0 22 mm ID, film thickness 0 2 μm The eluted compounds were detected by EIMS (70 eV, ion source temp 200°). The initial column temp (50°) was maintained for 2 min and then raised to 260° at 6°/min

*Blanks* Two blank expts were carried out to correct for the presence of contaminating halogenated compounds in the chemicals used (i) $CH_2Cl_2$ (3 l) and EtOAc (3 l) were evapd to a small vol on a rotary evaporator at 30° and analysed by GC-MS A small amount of $C_2Cl_4$ was detected in the $CH_2Cl_2$ concentrate, the EtOAc concentrate was free of contaminants (ii) Freshly prepared growth medium (4 5 l) was extd and analysed as described above The petrol fraction only showed solvent peaks The $CH_2Cl_2$ fraction contained 5-(1-hydroxyethyl)dihydro-2(3H)furanone and some nitrogen-containing compounds of unknown structure Samples of the EtOAc extract and the EtOAc-continuous extract only contained 5-(1-hydroxyethyl)dihydro-2(3H)furanone, which was also found in the $CH_2Cl_2$ extract These compounds most probably originate from the yeast extract in the growth medium.

*MS* The spectra of the six new halometabolites from *C. fumago* (**2–7**) are shown below, only peaks with intensities higher than 5% are given

2,2-*Dichloroethanol* (**2**) *m/z* (rel int.): 31 (100); other important peaks 29 (3), 43 (4), 48 (3), 49 (4), 79 (3), 83 (2)

1,3-*Dichloropropanol*-2 (**3**) *m/z* (rel int): 27 (6), 43 (42), 49 (7), 57 (9), 79 (100), 81 (32)

1,1,3-*Trichloropropanol*-2 (**4**). *m/z* (rel. int.). 27 (10), 29 (6), 43 (30), 49 (19), 51 (6), 77 (7), 79 (100; calcd for $C_2H_4^{35}ClO$ *m/z* = 78.9951, found 78 9954), 81 (31), 113 (17, calcd for $C_2H_3^{35}Cl_2O$

$m/z$ = 112 9560, found 112 9559), 115 (10, calcd for $C_2H_3^{35}Cl^{37}ClO$ $m/z$ = 114 9530, found 114 9525)

1,1,3,3-*Tetrachloropropanol*-2 (**5**) $m/z$ (rel int) 49 (32), 51 (12), 61 (6), 77 (38), 78 (6), 79 (12), 83 (18, calcd for $CH^{35}Cl_2O$ $m/z$ = 82 9455, found 82 9457), 85 (13), 113 (100, calcd for $C_2H_3^{35}Cl_2O$ $m/z$ = 112 9560, found 112 9560), 115 (62), 117 (10)

2-(3-*Chloro*-4-*hydroxyphenyl*)*ethanol* (**6**) $m/z$ (rel int) 51 (14), 77 (22), 105 (5), 107 (7), 141 (100, calcd for $C_7H_6^{35}ClO$ $m/z$ = 141 0106, found 141 0092), 142 (10), 143 (30), 172 (23, calcd for $C_8H_9{}^{35}ClO_2$ $m/z$ = 172 0291, found 172 0272), 174 (9)

2-(3,5-*Dichloro*-4-*hydroxyphenyl*)*ethanol* (**7**) $m/z$ (rel int) 31 (7), 51 (7), 75 (17), 111 (11), 141 (10), 175 (100, calcd for $C_7H_5^{35}Cl_2O$ $m/z$ = 174 9716, found 174 9705), 176 (12), 177 (63), 178 (8), 179 (11), 206 (25, calcd for $C_8H_8^{35}Cl_2O_2$ $m/z$ = 205 9900, found 205 9910), 208 (15)

*Chlorination of* 2-(4-*hydroxyphenyl*)*ethanol* (i) By chloroperoxidase (CPO) to a soln of 138 mg (1 mmol) of 2-(4-hydroxyphenyl)ethanol in 0 1 M $H_3PO_4$/KOH pH 2 7 containing 20 mM of KCl was added $H_2O_2$ in eight portions at 45 min intervals, and 300 $\mu$g of chloroperoxidase in three portions at 135 min intervals The mix was stirred for 1 day, the small amount of orange ppt that formed was discarded The mixt was extracted × 3 with $CH_2Cl_2$, the extract dried ($MgSO_4$) and the solvent evapd using a rotary evaporator The residue consisted of 2-(3-chloro-4-hydroxyphenyl)ethanol together with some of the 3,5-dichloro compound, as was demonstrated by NMR (90 MHz, $CDCl_3$–DMSO-$d_6$) (ii) By HOCl to a similar soln as described under (i) was added very slowly 186 $\mu$g (2 5 mmol) of NaOCl The reaction mixt was stirred for 6 hr at room temp and then extracted with $CH_2Cl_2$ The extract was dried ($MgSO_4$), evapd and analysed The product proved to be 2-(3-chloro-4-hydroxyphenyl)ethanol together with a small amount of the 3,5-dichloro compound, as was shown by NMR

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