New branches in the degradation pathway of monochlorocatechols by *Aspergillus nidulans*: a metabolomics analysis

Tiago M. Martins,[a] Oscar Núñez,[b] Hector Gallart-Ayala,[b] Maria Cristina Leitão,[a] Maria Teresa Galceran,[b] and Cristina Silva Pereira,[a*]

Research Highlights:

- One of the few studies on mono-chlorophenols/chlorocatechols degradation by fungi
- *Aspergillus nidulans* showed major dechlorination paths and conjugation reactions
- 3-Chlorodienelactone and catechol are key intermediates in unknown degradation paths
- The new fungal degradation paths might avoid known bacterial dead-ends

*Novelty Statement

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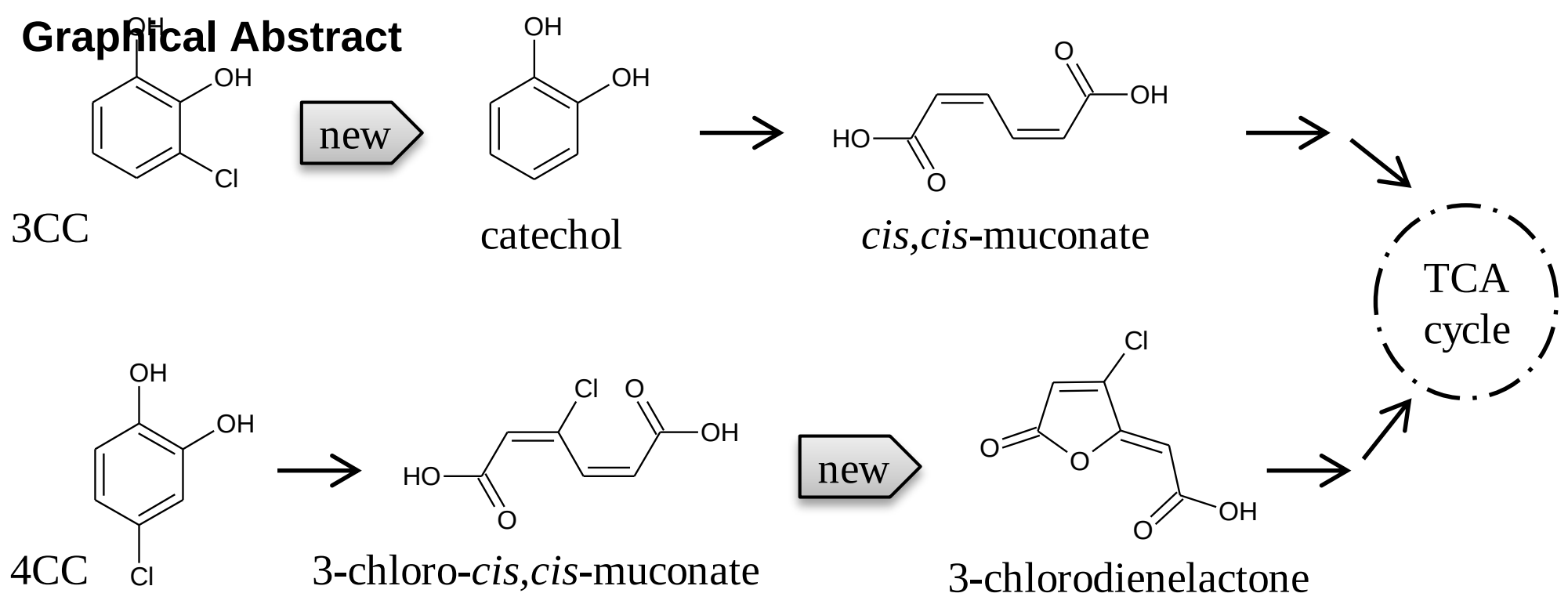
Novelty Statement: The present contribution focus on monochlorocatechols degradation by fungi. Its significance is highlighted by several facts: monochlorocatechols are key degradation intermediates of numerous chlorinated aromatic hydrocarbons (i), which are critical environmental pollutants (ii) but fungi role in their environmental mitigation remains largely ignored (iii). We believe this is of high relevance for the readers of the Journal of Hazardous Materials, particularly in the topics of biological degradation and environmental fate of critical pollutants. Our original data made apparent that fungi strongly influence the toxic-derived metabolome, ensuring unique degradation paths which might complement and/or compete with bacteria activity.

Graphical Abstract



*Abstract

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Abstract

A collective view of the degradation of monochlorocatechols in fungi is yet to be attained, though these compounds are recognised as key degradation intermediates of numerous chlorinated aromatic hydrocarbons, including monochlorophenols. In the present contribution we have analysed the degradation pathways of monochlorophenols in *Aspergillus nidulans* using essentially metabolomics. Degradation intermediates herein identified included those commonly reported (*e.g.* 3-chloro-*cis*,*cis*-muconate) but also compounds never reported before in fungi revealing for 4-chlorocatechol and for 3-chlorocatechol unknown degradation paths yielding 3-chlorodienelactone and catechol, respectively. A different 3-chlorocatechol degradation path led to accumulation of 2-chloromuconates (a potential dead-end), notwithstanding preliminary evidence of chloromuconolactones and protoanemonin simultaneous formation. In addition, some transformation intermediates, of which sulfate conjugates of mono-chlorophenols/chlorocatechols were the most common, were also identified. This study provides critical information for understanding the role of fungi in the degradation of chlorinated aromatic hydrocarbons; furthering their utility in the development of innovative bioremediation strategies.

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A collective view of the degradation of monochlorocatechols in fungi is yet to be attained, though these compounds are recognised as key degradation intermediates of numerous chlorinated aromatic hydrocarbons, including monochlorophenols. In the present contribution we have analysed the degradation pathways of monochlorophenols in *Aspergillus nidulans* using essentially metabolomics. Degradation intermediates herein identified included those commonly reported (*e.g.* 3-chloro-*cis*,*cis*-muconate) but also compounds never reported before in fungi revealing for 4-chlorocatechol and for 3-chlorocatechol unknown degradation paths yielding 3-chlorodienelactone and catechol, respectively. A different 3-chlorocatechol degradation path led to accumulation of 2-chloromuconates (a potential dead-end), notwithstanding preliminary evidence of chloromuconolactones and protoanemonin simultaneous formation. In addition, some transformation intermediates, of which sulfate conjugates of mono-chlorophenols/chlorocatechols were the most common, were also identified. This study provides critical information for understanding the role of fungi in the degradation of chlorinated aromatic hydrocarbons; furthering their utility in the development of innovative bioremediation strategies.

Keywords: chlorinated aromatic hydrocarbons, monochlorophenols, monochlorocatechols, *Aspergillus nidulans*, biodegradation, metabolic pathway

Introduction

Chlorinated aromatic hydrocarbons are widely distributed in the environment and many are highly toxic and persistent [1,2]. Globally, their environmental dissemination is a consequence of their extensive use in man´s activities (*e.g.* pesticides, solvents and plastics [1,2]), notwithstanding that some can be also produced in natural processes [3,4]. Their toxicity may increase as a result of degradation, either abiotic or biotic [5-7]. Photolysis has been shown to increase the toxicity of monochlorophenols (mCPs) due to transformation into the corresponding chlorobiphenyls [6]. Biodegradation of substituted derivatives of biphenyls has been shown to produce chlorinated phenyl lactones as dead-end metabolites [8,9]. Even bacterial degradation of the least chlorinated and less toxic phenols may end in the accumulation of several dead-end metabolites, including the antibiotic protoanemonin or its chlorinated derivative [7,10].

Degradation of monochlorinated aromatic hydrocarbons by bacteria usually starts with oxidation to monochlorocatechols (mCCs), which are in turn subsequently oxidised to chloromuconates [11,12]. Further degradation and concomitant dehalogenation, may occur by isomerisation of the latter [10]. Nevertheless, chlorocatechols often accumulate or are transformed into toxic dead-end metabolites [7,10]. This is currently considered to be a major limitation to bacterial degradation of chlorinated aromatic hydrocarbons [7,13]. Nevertheless, fungi, which are one of the ubiquitous life forms on earth, are known to play a fundamental role in the degradation of toxics [14,15]. They have already been shown to degrade numerous chlorinated aromatic hydrocarbons forming as key intermediates chlorocatechols, in a similar mode to that observed in bacteria. Accordingly, chlorocatechols have been detected in fungal cultures during degradation of *e.g.* chlorophenols [16-18], chlorobenzenes [19] and polychlorinated dibenzo-*p*-dioxins [20]. However, the degradation kinetics of mCPs

varies between fungal species [17,18] and has been shown to correlate with the specific activity of phenol monooxygenase against these compounds [17]. *Phanerochaete chrysosporium* degrades 2-chlorophenol (2CP) faster than 3-chlorophenol (3CP) and 4-chlorophenol (4CP) [18]. In opposition, *Penicillium frequentans* degrades the last two compounds faster than the former [17]. Multiple hydroxylation of 3CP or 4CP was shown to occur sequentially and rather independently of the position of the chlorine atom in cultures of *Penicillium simplicissimum* [21]. In contrast this strain was unable to hydroxylate 2CP. Other studies have shown that in cultures of *P. frequentans* or *Candida maltosa*, 2CP and 4CP yielded respectively 3-chlorocatechol (3CC) and 4-chlorocatechol (4CC), which in turn led to formation of either the dead-end metabolite 2-chloromuconate or dienelactone, whereas 3CP transformation combined these two pathways [17,22]. Similarly, degradation of 4CP by *A. nidulans* proceeded by transformation to 4CC and two other unknown compounds [23]. Mineralisation of mCPs by fungi is regarded as impossible/improbable on the basis of chloride release estimations [17,21-23]. Most recent studies focus on the degradation pathways upstream those of mCCs [1,2,14], hence, a collective view of their degradation intermediates and pathways is still missing.

The aforementioned inspired the present work, which aims to elucidate the degradation pathway of mCPs and mCCs in *A. nidulans*. The data obtained here provide critical information for understanding the role of fungi in the degradation of chlorinated aromatic hydrocarbons in the environment.

Experimental procedures

Chemicals

Grade chemicals: If not explicitly stated otherwise, chemicals were of analytical grade and purchased from Sigma-Aldrich, with the exception of acetonitrile and methanol

(Fisher Scientific), chlorophenols (Riedel-de Haën) and *trans*-acetylacrylate (Alfa Aesar).

Synthesised chemicals: *cis*-Acetylacrylate; acetylacrylate acylale; protoanemonin and 3-chloro-*cis*,*trans*-muconate, *cis*- and *trans*-dienelactone and 3-chloromuconolactone were obtained using defined protocols, respectively: UV isomerisation of *trans*-acetylacrylate [24]; tautomerisation at low pH of *cis*-acetylacrylate [25]; acetylation of *trans*-acetylacrylate [26] and isomerisation and lactonisation of 3-chloro-*cis*,*cis*-muconate [27]. *cis*,*cis*-Muconate and 3-chloro-*cis*,*cis*-muconate were synthesised respectively from catechol and 4CC using a crude enzyme extract with dioxygenase activity [28] (see below).

Strain, media and growth conditions

The reference strain *A. nidulans* FGSC A4 was used in all studies. Fungal cultures were inoculated with conidia (10^5 conidia/mL, prepared as previously described [29]) in 250 mL screw thread flasks with a working volume of 50 mL (minimal media (MM) [29] containing 10 g/L glucose) and incubated at 27 °C and 90 rpm, in the dark.

Chlorophenols toxicity tests and degradation experiments

Inhibition of *A. nidulans* spore germination and growth (minimal inhibitory concentration, MIC) in media containing defined concentrations of mCPs was monitored daily using a micro-cultivation approach [30], except for 2CP, which at 25 °C has a vapour pressure 20 fold higher than the other mCPs [31], where 40 mL screw thread flasks with a working volume of 15 mL were used instead.

To test *A. nidulans* ability to degrade the mCPs, four days old batch cultures grown in glucose MM, were spiked with 0.8 mM of each monochlorophenol (500 fold concentrated stocks in ethanol) and further incubated for fourteen days. Cultures without mCPs and controls for abiotic decay were also prepared. Aliquots of 200 µL of

the culture media were taken at defined time points. At the end of incubation the culture media was filtered (glass fibre filters, Millipore) and both the filtrate and the mycelia were stored at −80 °C until further analysis. Two independent experiments were analysed each with three biological replicates.

Detection, quantification and identification of monochlorophenols degradation intermediates

Detection and quantification of mCPs (or their degradation products) in either the culture media or in the extracellular organic extracts (*ca*. 100 fold concentrated) was done as previously described [32]. Comparison with standards was used to identify/quantify the target compounds (Table 1), with the exception of 3CC that was inferred thought comparison with 4CC (similar RT_{LC} and λ_{max}). Compounds X211, X280a and X280b were manually collected in the chromatographic eluent. These aliquots were first dried and extracted [32] and their purity/identity evaluated.

UHPLC-HRMS analyses of the organic extracts (identification of additional degradation intermediates) were operated in negative ESI mode using a Q-Exactive Orbitrap MS system (Thermo-Fisher) as previously described [33]. MS data was processed by ExactFinder^{TM} 2.0 software (Thermo-Fisher) by applying a user target database list and validated, whenever possible, using standard compounds.

Kinetic and data analysis

Monochlorophenol decay functions were fitted by means of linear and nonlinear least square regression and compared for the best fit with GraphPad Prism 5 software, essentially as previously described [34].

Enzymatic transformation of catechols

To prepare a crude enzyme extract with dioxygenase activity, *A. nidulans* mycelia (grown for seven days in 50 mM sodium salicylate MM, *n.b.* specific activity was

approximately 300 fold higher when using sodium salicylate MM instead of glucose MM) was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle; homogenised in extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 20 µM $FeSO_4$ and 0.1 mM AEBSF; 5 ml to 1 g); incubated in ice for 30 min and clean from the cell debris by centrifugation (10.000*g*, 20 min, 4 °C). The crude enzyme extract (buffer exchanged 5 times using 10k MWCO Vivaspin columns, Sartorius) was used immediately or stored at –20 °C. Dioxygenase activity was determined by measuring the rate of muconate accumulation at 260 nm [10] using catechol or 4CC as substrates. Enzymatic transformation of catechols was done by incubating at room temperature 0.2 mM of the respective catechol in 50 mM Tris-HCl, pH 8.0, with *ca.* 150 U/mg.mL of catechol 1,2-dioxygenase or *ca.* 10 U/mg.mL of 4-chlorocatechol 1,2-dioxygenase. A total of 2 mM of each catechol was added by manually spiking the reaction at regular intervals for preventing substrate inhibition. The reaction was monitored by UPLC until complete enzymatic turnover of 4CC to 3-chloro-*cis*,*cis*-muconate (*ca.* 3 days). At this point the reaction medium was first acidified to pH<1, then extracted [32] and finally analysed by UHPLC-HRMS.

Results

Decay of monochlorophenols during *A. nidulans* growth

2CP, 3CP and 4CP MICs on *A. nidulans* germination and growth were respectively >1.00, 1.00 and 0.85 mM. Accordingly at an initial concentration of 0.80 mM, all the mCPs were completely transformed by the fungus within the fourteen days of cultivation (Fig. 1a). Their degradation followed a sigmoid decay response for 2CP and 3CP and an exponential decay response for 4CP (Table S1). Preliminary data indicated that mycelial bio-accumulation was negligible (data not shown). mCPs abiotic degradation in the controls was below 10% at the end of fourteen days.

Quantification of monochlorophenols degradation intermediates

The mCPs degradation intermediates produced by *A. nidulans* along the incubation time (UPLC analyses) are depicted in Table 1 (Fig. S1). Data revealed the transient accumulation of **monochlorocatechols** (Fig. 2c and d), as previously reported in other fungi [17,21-23]. 2CP and 4CP degradation pathways yielded essentially **3CC** and **4CC** (Fig. 1c and d) respectively and that of 3CP yielded the two former mCCs (Fig. 1c and d). 3CC and 4CC were completely transformed at the same time point as their corresponding parent compounds (Fig. 1a, c and d). The formed 4CC was further metabolised to **3-chloro-*cis,cis*-muconate** (Fig. 1b), which accumulated at a similar rate to a compound herein named as **X211** (λ_{max} 211 nm) (Fig. 1h). As the concentration of both decreased, ***trans*-acetylacrylate** accumulated (Fig. 1f). In the 4CP cultures, ***cis*-dienelactone** was also detected in small quantities (not quantified).

Near the end of the incubation period the concentration of all the identified degradation intermediates of 3CP and 4CP decreased, except that of *trans*-acetylacrylate. Its MIC was at least 500 fold higher than the maximum concentration detected (10 mM < MIC ≤ 15 mM). *trans*-Acetylacrylate was fully co-metabolised in glucose MM within two weeks (5 µM to 2 mM) and used as a sole carbon and energy source (5 and 10 mM). Albeit in these cultures no degradation intermediates of *trans*-acetylacrylate could be detected by UPLC.

3CC presumably yielded **X280a** and **X280b** (λ_{max} *ca.* 280 nm), which showed a late accumulation profile (Fig. 1g). 2-Chloro-*cis*,*cis*-muconate, relatively stable in mild acidic conditions [35] with a λ_{max} 275 nm [36], has been systematically reported as the most likely ring cleavage product of 3CC [17,22,37,38]. In an acidic solution the isolated compound X280a was efficiently converted to X280b; a conversation that also occurred, yet slowly, upon acidification of the culture media containing X280a. 2-

Chloro-*cis*,*cis*-muconate yields 2-chloro-*cis*,*trans*-muconate at pH<1 [11]. Thus, X280a and X280b can be preliminary identified, respectively, as **2-chloro-*cis*,*cis*-muconate** and **2-chloro-*cis*,*trans*-muconate**. Finally, another common degradation intermediate of 2CP and 3CP was ***cis*,*cis*-muconate** that accumulated in low amounts (<5 µM) after fourteen days of incubation (Fig. 1e). This compound has never been identified as a degradation intermediate of monochlorophenols in fungi. In the abiotic controls only mCCs (<2 µM) were detected (Table 1).

Identification of novel monochlorocatechols degradation intermediates

Aiming to resolve the identity of the aforementioned unidentified compounds and to indentify new ones, the culture extracts (*ca.* 100 fold concentrated) were analysed by UHPLC-HRMS (Table 2, Table S2 and Fig. S2). The identified compounds can be divided in two major groups, namely conjugated or oxidised forms of mCPs and mCCs and chlorinated or non-chlorinated compounds which resulted from the degradation of the mCCs. The extracts were re-analysed by UPLC (Table 1) to verify the existence of abiotic transformations which might occur during acidification (*ca.* pH 2) [32].

Several compounds with the same accurate ion mass of **chloromuconate** or **chloromuconolactone** were detected (Table 2). For each of these compounds several isomers are possible. In the 3CP and 4CP cultures, **3-chloro-*cis*,*trans*-muconate** and ***cis*-dienelactone** were systematically identified. While the last was previously detected by UPLC either in the culture media or in the corresponding extracts, the first was only detected in the culture extracts (Table 1). The 3-chloro-*cis*,*trans*-muconate identified here was produced by isomerisation of 3-chloro-*cis*,*cis*-muconate, a very favourable reaction in an acidified media [27]. In addition, *cis*-dienelactone, in part formed during the degradation of mCPs (Table 1), was also produced by the lactonisation of 3-chloro-*cis*,*cis*-muconate upon media acidification [27]. It should be stated that only for pH<1,

3-chloro-*cis,cis*-muconate would also yield 3-chloromuconolactone [27]; hence not likely in the conditions used here.

3-Chlorodienelactone ($[M-H]^-$ *m/z* 172.9647 and $[M-CO_2-H]^-$ *m/z* 128.9749), detected in the 3CP or the 4CP culture extracts, was demonstrated to be **X211** upon its isolation and analysis. Both extracts probably contained also **3-hydroxymuconolactone** ($[M-H]^-$ *m/z* 157.0142), which was detected after neutralisation of a 3-chloromuconolactone solution (Table 2); a very favourable reaction at neutral pH [39]. As expected, the data confirmed also the presence of ***trans*-acetylacrylate** ($[M-H]^-$ *m/z* 113.0244).

X280b was confirmed as **2-chloro-*cis,trans*-muconate** upon its isolation and analysis; which was systematically detected in the 2CP and the 3CP culture extracts ($[M-H]^-$ *m/z* 174.9804, λ_{max} *ca.* 280 nm). In the 3CP culture extracts, in addition to 3-chloro-*cis,trans*-muconate ($RT_{LC-HRMS}$ = 4.30) and 2-chloro-*cis,trans*-muconate ($RT_{LC-HRMS}$ = 3.67 min), three other compounds with the same accurate ion mass, yet distinct retention times ($RT_{LC-HRMS}$ = 2.23, 3.33 or 4.04 min), were also detected. The compounds eluting at 3.33 or 4.04 min showed a accumulation profile along the incubation time similar to that observed for X280a (Fig. 1g). Based on their ion mass fragments produced at the ionisation source they are likely chloromuconates, one a **2-chloro-*cis,cis*-muconate** (X208a) and the other either **2-** or **3-chloro-*trans,trans*-muconate**. Finally, the compound eluting at 2.23 (only detected in the first time points) fits the description of a chloromuconolactone. Most likely it is either **2-** or **5-chloromuconolactone**, since 3-chloromuconolactone (standard available) was not detected and 4-chloromuconolactone has been described as unstable [10,40].

2CP and 3CP culture extracts were shown to contain also **catechol** (UHPLC-HRMS) - the most likely precursor of ***cis,cis*-muconate** (Fig. 1e, Table 1). In the 2CP

culture extract collected after eight days of incubation, one compound showed similarity with the standard of **protoanemonin** ($[M-H]^-$ *m/z* 95.0139, $RT_{LC-HRMS}$ = 1.34 min); however low ionisation and retention time hampered validation.

Other classes of compounds detected in the culture extracts included **sulfate conjugates** of all **mCPs** and **mCCs** (Table 2). Other two putative conjugates of mCPs with accurate ion masses of *m/z* 199.0171 and 229.0273 (and *m/z* 126.9956, which corresponds to mCP), were detected in the 3CP and the 4CP culture extracts. In addition, **chlorotrihydroxibenzene**, a more hydroxylated form of chlorocatechol, was detected in both extracts at the time points of incubation where 4CC accumulated sufficiently (Table 2). In the 2CP culture extracts an isomer of 2CP such as chlorobenzene oxide was also detected ($[M-H]^-$ *m/z* 126.9956, $RT_{LC-HRMS}$ = 4.90). In all culture extracts, as well as in the respective abiotic controls, **chlorobenzoquinone** was putatively detected at the eighth day of incubation, most likely corresponding to the oxidised form of the chlorocatechol detected in the same extract (Table 2).

Transformation of 4-chlorocatechol by *A. nidulans* mycelial protein extracts

4CC was readily converted to 3-chloro-*cis,cis*-muconate when incubated with a crude enzyme extract with dioxygenase activity (Fig. S3, Table S3). The corresponding organic extracts (*ca*. 50 fold concentrated) showed the same degradation intermediates detected by UHPLC-HRMS in the 4CP culture extracts (Table 2), as well as two additional compounds, namely 3-chloromuconolactone ($[M-H]^-$ *m/z* 174.9804) and chloromaleylacetate ($[M-H]^-$ *m/z* 190.9753) (Table S3). 3-chloromuconolactone formation occurred due to the intentional use of pH<1; conditions where 3-chloro-*cis,cis*-muconate yields 3-chloro-*cis,trans*-muconate, *cis*-dienelactone and 3-chloromuconolactone [27]. The detection of chloromaleylacetate, a likely degradation

product of 3-chlorodienalactone, further corroborates the identity of X211 as the former compound.

Discussion

The selection of *A. nidulans*, a reference strain, should reflect a pollutant unbiased environmental background. This opposes to the recurrent selection of environmental strains showing high degradation ability [17,21,22]. Analysis of 2CP and 3CP degradation profiles (Fig. 1a, Table S1) showed that after an initial lag phase (probably for induction of degradation enzymes) [41], accumulation of inhibitory degradation intermediates, if any, was inconsequential. On the contrary, during 4CP degradation, no lag phase could be observed, yet some inhibitory degradation intermediates likely accumulated, one of which was probably 3-chloro-*cis,cis*-muconate (Fig. 1b). Its accumulation during 4CP degradation was *ca*. 3 fold higher than during 3CP degradation. Phenol 2-monooxygenases (EC 1.14.13.7), of which the putative encoding genes are expressed in *A. nidulans* cultures under broad conditions [42], have been shown to hydroxylate preferentially 4CP>3CP>>2CP [17,43] corroborating these findings. 4CC was also hydroxylated yielding chlorotrihydroxibenzene (Table 2) as previously reported for other Ascomycota [21]. Levels were very low, probably due to reduced phenol 2-monooxygenase activity against catechols [44].

Pathway branch in 4-chlorocatechol degradation yielding 3-chlorodienelactone

In *A. nidulans* cultures the mCCs formed during the degradation of 3CP or 4CP were further degraded through distinct pathways, each leading to further transformations through multiple steps, either biotic or abiotic (Fig. 2). The proposed pathway disagrees from the simplistic view generally reported in the literature. The degradation intermediates of 4CC included those typically reported, namely 3-chloro-*cis,cis*-

muconate and *cis*-dienelactone, as well as some never observed previously in fungi, namely 3-chlorodienelactone and *cis*- and *trans*-acetylacrylate (Table 1 and 2).

3-Chloro-*cis*,*cis*-muconate was likely converted to 3-chloromuconolactone (Fig. 2) *via* 1,4-cycloisomerisation. Similar reactions have been reported in *Aspergillus niger* [45]. Such reaction might either involve a muconate lactonising enzyme (EC 5.5.1.1) (well characterise in the yeast *Trichosporon cutaneum* [39]) or a carboxy muconate cyclase (EC 5.5.1.5), which in *Neurospora crassa* have been shown to retain residual, yet significant, muconate lactonising activity over *cis*,*cis*-muconate [46]. Both enzymes are associated with the β-ketoadipate pathway, yet belonging respectively to the catechol and protocatechuate branches [46]. The *N. crassa* carboxy muconate cyclase gene shares high homology to a single *A. nidulans* gene (AN1151). Up to now, none of these enzymes has been fully characterised in a particular species, but it is well accepted that both catalyse the formation of 3- derivatives of muconolactones. 3-Chloromuconolactone was not detected in any of these cultures, probably because it was either rapidly hydrolysed abiotically at neutral pHs to 3-hydroxymuconolactone [39] or isomerised enzymatically to 3-chlorodienelactone through a pathway branch that might differ from that yielding *cis*-dienelactone (Fig. 2). 3-Chlorodienelactone concentration profile along time (Fig. 1h) suggests that its degradation likely yielded the chlorinated intermediate chloromaleylacetate, as previously reported for other chloro and methyl derived dienelactones [10,47]. Chloromaleylacetate was also formed upon 4CC incubation with the crude enzyme extract with dioxygenase activity (Table S3). Notwithstanding, 2-chloromaleylacetate has been shown to be formed abiotically at neutral pH from 2-chloro-*cis*-dienelactone [10].

The *cis*-*trans* isomerisation of acetylacrylate is catalysed by maleylacetoacetate isomerase (EC 5.2.1.2) [48] which participates in the homogentisate pathway of *A.*

nidulans [49]. Likely the spontaneous decarboxylation of maleylacetate originated the *cis*-acetylacrylate [12] which in turn yielded *trans*-acetylacrylate (Fig. 1f). The first reaction at neutral conditions competes with maleylacetate reductase (EC 1.3.1.32) [50] that would form 3-oxoadipate (β-ketoadipate) instead. In addition, *cis*-Acetylacrylate has been previously described as an intermediate in the degradation of mCPs by bacteria [51], in general, yielded through protoanemonin hydrolysis [52].

3-Chloro-*cis,cis*-muconate abiotic transformation to *cis*-dienelactone, through the 4-chloromuconolactone intermediate, at neutral pH (conditions used herein) is not likely to occur [27]. One cannot disregard that other mechanisms of *cis*-dienelactone formation might exist.

Overall the obtained data support that *A. nidulans* transformed 3-chloro-*cis,cis*-muconate through a 1,4-cycloisomerisation, yielding 3-chlorodienelactone. This branch has never been reported before in fungi probably due to the systematic use of acidic conditions which favours the abiotic formation of *cis*-dienelactone, *e.g.* pH 4.5 [18,22], 5.0 [23] and 5.6 [21]. Apart from the present study, only one used an alkaline growth media, pH 7.8 [17], yet the degradation products were not comprehensively characterised.

Pathway branch of 3-chlorocatechol yielding catechol

The downstream degradation intermediates of 3CC (Fig. 1c) included catechol and its respective ring fission product *cis,cis*-muconate (Table 1 and 2). Catechol is a preferred substrate for the catechol 1,2-dioxygenase activity (EC 1.13.11.1) detected in crude enzyme extracts of *A. nidulans* (Fig. S3) and previously reported *e.g.* in *P. frequentans* [17]. Most likely the degradation of *cis,cis*-muconate involved the catechol branch of the β-ketoadipate pathway [46]. This has never been reported in fungi before, probably because catechol and *cis,cis*-muconate concentrations in media were very low. Some

unresolved aspects deserve further investigation, especially the identity of the chlorinated-precursor yielding catechol. One can, however, assume that it might have been produced through reductive dehalogenation of 3CC [53] - the only common chlorinated aromatic intermediate in the 2CP and 3CP cultures.

Are the 2-chloromuconates a dead-end in 3-chlorocatechol degradation?

2-Chloro-*cis*,*cis*-muconate (X280a), herein detected in the 2CP and 3CP cultures (Fig. 1g, Table 1 and 2) has been previously regarded as a dead-end metabolite during 2CP degradation by other Ascomycota [17,22]. The activity of the muconate lactonising enzyme is affected by the chloride atom, particularly inhibited when present at the C2 position [17]. 2-Chloro-*cis*,*cis*-muconate and 2-chloro-*cis*,*trans*-muconate (X280b) concentrations in media reached a plateau after some cultivation time (Fig. 1g). The interconversion between the two isomers is probably catalysed by a maleylacetoacetate isomerase as previously reported [54].

Several compounds with the accurate ion mass of chloromuconolactone were detected (Table 2), although their identities remains uncertain. This suggests that degradation of 2-chloro-*cis*,*cis*-muconate to a chloromuconolactone (either the 2 or the 5) might have occurred. The chemical equilibrium of the interconversion of 2-chloro-*cis*,*cis*-muconate and the corresponding chloromuconolactones has been reported before in bacteria [37,38]. Some bacteria can further degrade the 2- or 5-chloromuconolactone yielding respectively protoanemonin or dienelactone [37]. In the 2CP cultures protoanemonin was putatively identified (Table 2); which likely yielded *cis*-acetylacrylate, either abiotically or enzymatically. The last reaction would involve a dienelactone hydrolase (EC 3.1.1.45) [52] (*A. nidulans* owns at least five encoding genes have been putatively annotated [55]). The converted *cis*-acetylacrylate would be in turn completely degraded by the fungus. In fact, in fourteen days, protoanemonin

was completely degraded biotically, while abiotically its degradation reached >60% and both acetylacrylate isomers accumulated in media.

Alternative paths to the degradation of monochlorophenols: conjugation reactions

Apart from the degradation intermediates of mCPs herein detected (at the heart of this study), some transformation intermediates (compounds involving oxidation and conjugation reactions) were also observed. Conjugation reactions are well established as common intracellular mechanisms of detoxification [32]. Not surprisingly, several conjugates of mCPs and mCCs were detected (Table 2), of which sulfate conjugates were the most frequent. Sulfate conjugation of several chlorinated aromatic hydrocarbons has been previously reported in fungi, including *Aspergillus* strains [32,56]. None of the other typically reported conjugates, including glutathione, glucuronide, glucose, ribose or methoxy conjugates [57] could be detected. Though other putative mCPs transformation intermediates were suggested, their identification requires further investigation.

Conclusions

Chlorinated aromatic hydrocarbons degradation by fungi have been largely ignored but well studied in bacteria [1]. Challenged by this, we resolved here major monochlorocatechols degradation paths through a comprehensive analysis of the degradation intermediates being formed in *A. nidulans* cultures. These, which included those commonly reported, but also some never observed before, revealed for 4CC and 3CC new degradation paths yielding 3-chlorodienelactone and catechol, respectively. Data corroborated previous finding that enzymes mediating lactonisation of chloromuconates in fungi (1,4-cycloisomerisation) differ from their bacterial counterparts (3,6-cycloisomerisation). This give fungi the capacity to circumvent the formation of dead-end metabolites in specific conditions, *e.g.* avoiding accumulation of

4-methylmuconolactone, which in bacteria is a dead-end metabolite produced during the degradation of *p*-cresol [58]. In addition, a different 3CC degradation path in *A. nidulans* led to accumulation of 2-chloromuconates (a potential dead-end), notwithstanding preliminary evidence that chloromuconolactones and protoanemonin formation also occurred. The last, regarded as a dead-end in bacteria [37], was also degraded by the fungus. This study reinforces the idea of the superior bioremediation capacities of filamentous fungi, in particular of Ascomycota [14]. They are expected to play major roles in the environmental mitigation of chlorinated aromatic hydrocarbons, especially in the soil ecosystem where they are main colonisers. If present, the spectra of biochemical reactions which fungi might undertake will strongly influence the toxic-derived metabolome, ensuring degradation paths which might complement and/or compete with bacteria activity.

Tables and Figures Captions

Table 1. Monochlorophenols degradation intermediates in *A. nidulans* cultures as detected by UPLC. The retention times (RT_{LC}) and the absorbance maxima (λ_{max}; maximum in bold) of the identified compounds (✓) are depicted.

Table 2. Monochlorophenols degradation intermediates in *A. nidulans* culture extracts detected by UHPLC-HRMS. For each compound the elemental chemical formula, the retention time ($RT_{LC\text{-}HRMS}$) and the expected mass in negative mode are indicated. For matters of simplicity, only compounds reporting an error lower than 5ppm, an isotopic pattern score higher than 90%, and a low noise/signal ratio were considered (✓).

Fig. 1. Time course for the degradation of mCPs and of some degradation intermediates in *A. nidulans* cultures (UPLC analyses). 2CP, 3CP and 4CP (A); 3-chloro-*cis*,*cis*-muconate (B), 3CC (C), 4CC (D), *cis*,*cis*-muconate (E), *trans*-acetylacrylate (F), X280a

and X280b (G), and X211 (H). Values represent means ± standard deviation of three biological replicates.

Fig. 2. Proposed pathway for the degradation of mCPs in *A. nidulans*. Compounds herein detected are underlined. Enzymatic and abiotic steps are indicated by single and double lined arrows.

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Table 1.

Compound	Chemical formula	λmax	RT_{LC} (min)	2CP	3CP	4CP
hydroxyquinol	$C_6H_6O_3$	**194**; 289	1.25			
cis-acetylacrylate	$C_5H_6O_3$	**196**	1.55		✓	✓
hydroquinone	$C_6H_6O_2$	**193**; 222; 290	1.58			
trans-dienelactone	$C_6H_4O_4$	**276**	1.64			
trans-acetylacrylate	$C_5H_6O_3$	**221**	1.73		✓	✓
cis,cis-muconate	$C_6H_6O_4$	**260**	1.78	✓	✓	
2-hydroxy-1,4-benzoquinone	$C_6H_4O_3$	191; **256**; 380	1.82			
3-chlorodienelactone	X211[d]	**211;** 304	2.02		✓	✓
3-chloromuconolactone	$C_6H_5ClO_4$	**216**	2.05			
2-chloro-*cis,cis*-muconate	X280a [d]	199; **278**	2.11	✓	✓	
cis-dienelactone	$C_6H_4O_4$	202; **276**	2.20			✓
3-chloro-*cis,cis*-muconate [a]	$C_6H_5ClO_4$	**207**; 257	2.27		✓	✓
protoanemonin	$C_5H_4O_2$	**260**	2.31			
chlorohydroquinone	$C_6H_5ClO_2$	**198**; 295	2.46			
catechol	$C_6H_6O_2$	**195**; 276	2.47			
2-chloro-*cis,trans*-muconate	X280b [d]	204; **279**	2.49	✓	✓	
3-chloro-*cis,trans*-muconate [b]	$C_6H_5ClO_4$	203; **266**	2.83		✓	✓
phenol	C_6H_6O	**193**; 271	3.09			
3-chlorocatechol [c]	$C_6H_5ClO_2$	**199**; 278	3.17	✓	✓	
4-chlorocatechol [c]	$C_6H_5ClO_2$	**200**; 285	3.31		✓	✓
2-chlorophenol	C_6H_5ClO	**196**; 276	3.80	✓		
4-chlorophenol	C_6H_5ClO	**196**; 227; 282	3.92			✓
3-chlorophenol	C_6H_5ClO	**197**; 276	3.98		✓	

[a] only detected in aliquots of culture media; [b] only detected in the organic extracts; [c] also detected in abiotic controls; [d] unknown compound

Table 2.

Compound	Chemical formula	Expected ion mass	$RT_{LC\text{-}HRMS}$ (min)	2CP			3CP			4CP		
				2d	3d	8d	2d	3d	8d	2d	3d	8d
chlorinated or non-chlorinated compounds which resulted from the degradation of the chlorocatechols												
protoanemonin [a]	$C_5H_4O_2$	95.0139	1.33 – 1.35			✓						
3-hydroxymuconolactone	$C_6H_6O_5$	157.0142	1.31 – 1.44				✓	✓	✓	✓	✓	✓
trans-acetylacrylate [a]	$C_5H_6O_3$	113.0244	1.76 – 1.90					✓	✓			✓
3-chlorodienelactone	$C_6H_3ClO_4$	172.9647	2.01 – 2.15				✓	✓	✓	✓	✓	✓
chloromuconolactone	$C_6H_5ClO_4$	174.9804	2.29 – 2.32				✓	✓				
2-chloro-*cis,cis*-muconate	$C_6H_5ClO_4$	174.9804	3.31 – 3.33				✓	✓	✓			
cis-dienelactone [a]	$C_6H_4O_4$	139.0037	3.33 – 3.35					✓		✓	✓	✓
catechol [a]	$C_6H_6O_2$	109.0295	3.56		✓		✓	✓				
2-chloro-*cis,trans*-muconate	$C_6H_5ClO_4$	174.9804	3.67 – 3.70		✓	✓	✓	✓	✓			
chloromuconate	$C_6H_5ClO_4$	174.9804	4.04					✓	✓			
3-chloro-*cis,trans*-muconate [a]	$C_6H_5ClO_4$	174.9804	4.29 – 4.31				✓	✓	✓	✓	✓	✓
conjugated or oxidised forms of monochlorophenols and monochlorocatechols												
chlorotrihydroxybenzene	$C_6H_5ClO_3$	158.9854	4.11					✓		✓	✓	
2CP sulfate conjugate	$C_6H_5ClO_4S$	206.9524	4.08 – 4.12	✓	✓	✓						
3CP sulfate conjugate	$C_6H_5ClO_4S$	206.9524	4.07					✓				
4CP sulfate conjugate	$C_6H_5ClO_4S$	206.9524	3.99 – 4.19								✓	✓
3CC sulfate conjugate	$C_6H_5ClO_5S$	222.9473	4.33 – 4.38	✓	✓	✓	✓	✓				
4CC sulfate conjugate	$C_6H_5ClO_5S$	222.9473	4.36 – 4.44				✓	✓	✓	✓	✓	✓
3-chloro-1,2-benzoquinone [b, c]	$C_6H_3ClO_2$	186.9804	4.52 – 4.53	✓	✓	✓						
4-chloro-1,2-benzoquinone [b, c]	$C_6H_3ClO_2$	186.9804	4.61 – 4.62						✓			✓
3CC [b]	$C_6H_5ClO_2$	142.9905	4.61 – 4.62	✓	✓	✓	✓	✓	✓			

4CC [a, b]	$C_6H_5ClO_2$	142.9905	4.75 – 4.76				✓	✓	✓	✓	✓	✓
2CP isomer	C_6H_5ClO	126.9956	4.90 – 4.91	✓	✓	✓						
unknown conjugate	$C_{10}H_{11}ClO_4$	229.0273	5.09 – 5.10				✓	✓	✓	✓	✓	✓
unknown conjugate	$C_9H_9ClO_3$	199.0167	5.52 – 5.54				✓	✓	✓	✓	✓	✓
		parent compounds										
2CP [a]	C_6H_5ClO	126.9956	5.22 – 5.25		✓							
3CP [a]	C_6H_5ClO	126.9956	5.44 – 5.45				✓	✓				
4CP [a]	C_6H_5ClO	126.9956	5.38 – 5.39							✓	✓	

[a] the identification of these compounds was validated by the analysis of standard compounds; [b] also detected in the respective abiotic controls; [c] ion mass of the formic acid adduct

Figure

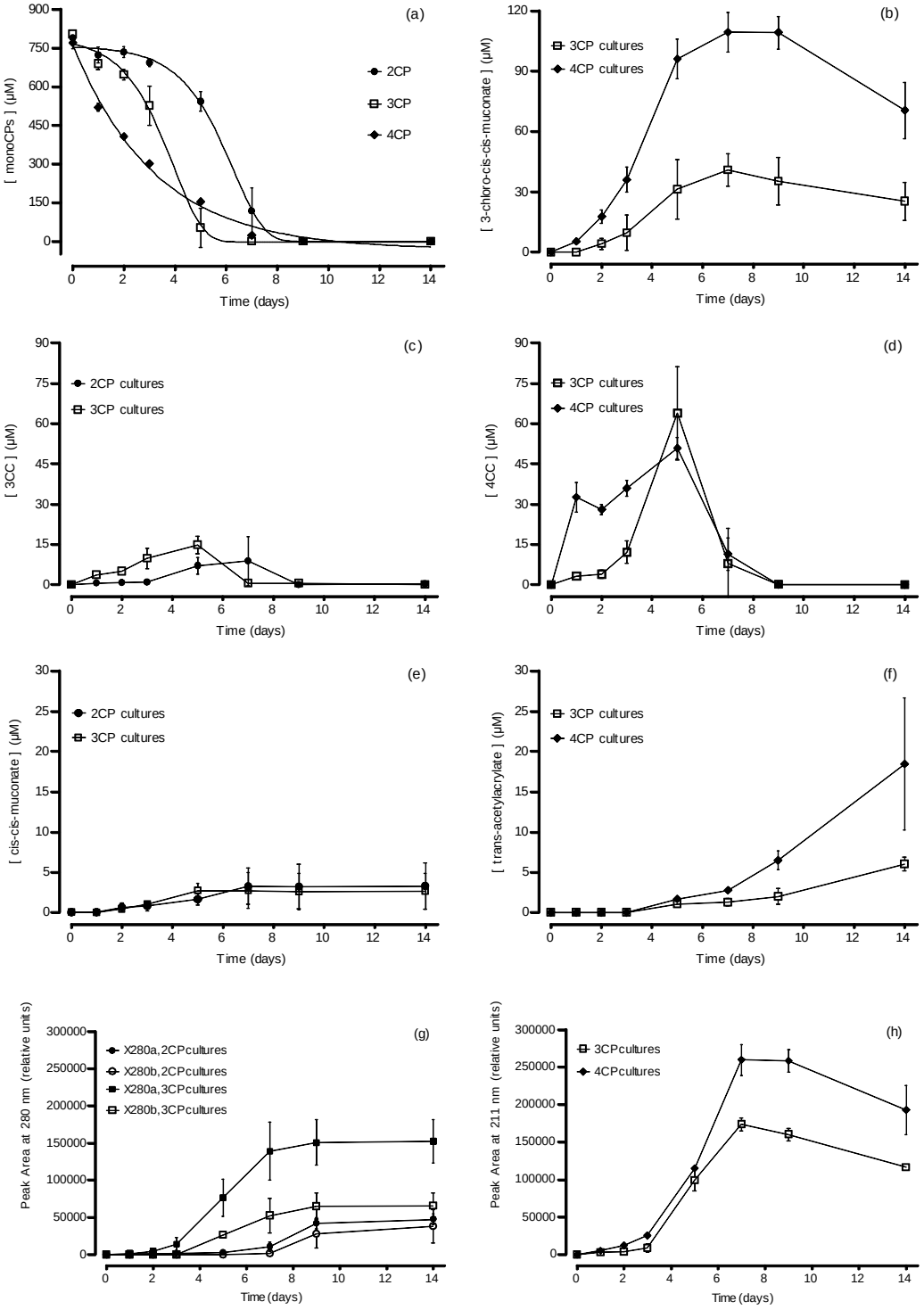
(a)
[monoCPs] (μM)
2CP
3CP
4CP
Time (days)
(b)
[3-chloro-cis-cis-muconate] (μM)
3CP cultures
4CP cultures
Time (days)
(c)
[3CC] (μM)
2CP cultures
3CP cultures
Time (days)
(d)
[4CC] (μM)
3CP cultures
4CP cultures
Time (days)
(e)
[cis-cis-muconate] (μM)
2CP cultures
3CP cultures
Time (days)
(f)
[trans-acetylacrylate] (μM)
3CP cultures
4CP cultures
Time (days)
(g)
Peak Area at 280 nm (relative units)
X280a,2CPcultures
X280b,2CPcultures
X280a,3CPcultures
X280b,3CPcultures
Time (days)
(h)
Peak Area at 211 nm (relative units)
3CPcultures
4CPcultures
Time (days)

Fig. 1.

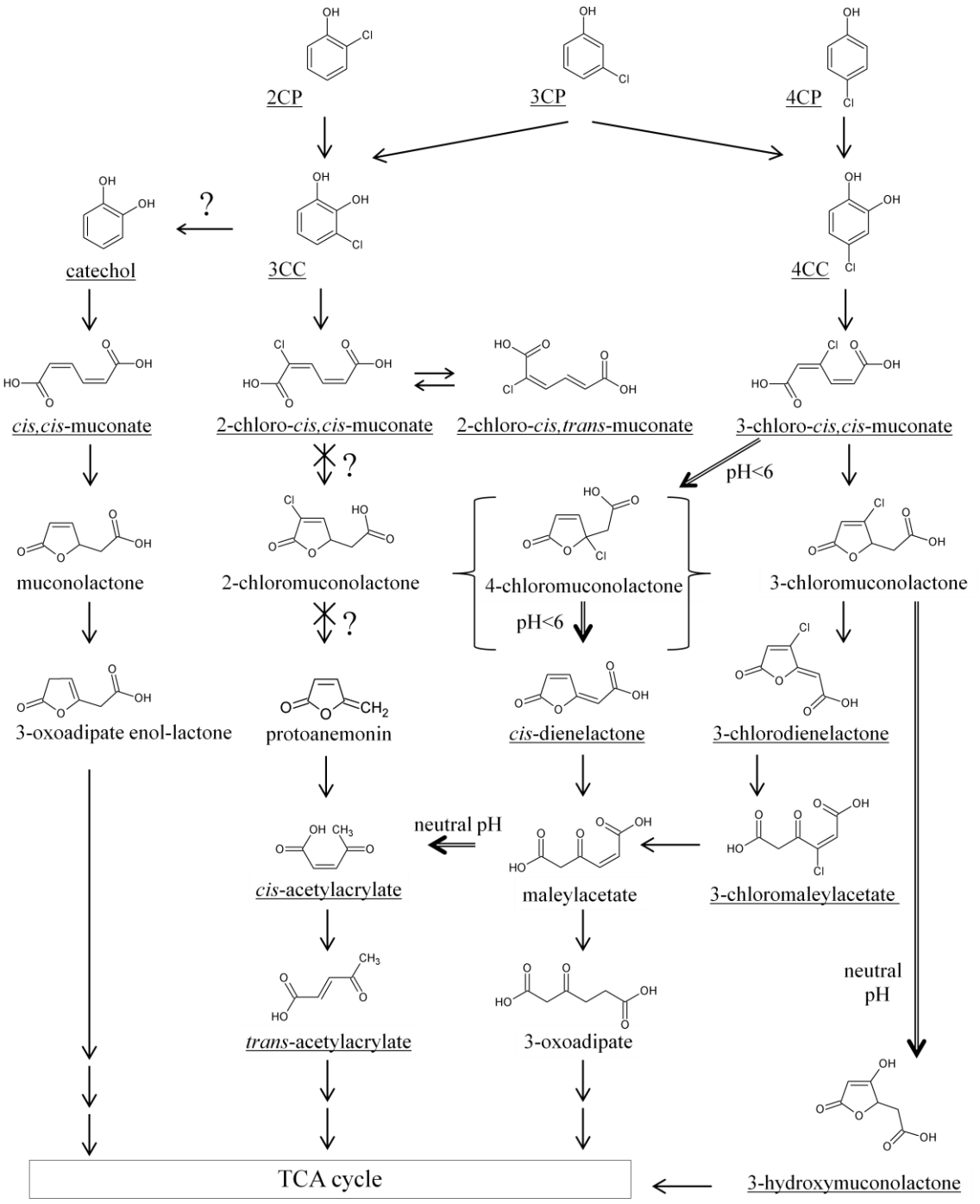
2CP
3CP
4CP
catechol
3CC
4CC
cis,cis-muconate
2-chloro-cis,cis-muconate
2-chloro-cis,trans-muconate
3-chloro-cis,cis-muconate
pH<6
muconolactone
2-chloromuconolactone
4-chloromuconolactone
3-chloromuconolactone
pH<6
3-oxoadipate enol-lactone
protoanemonin
cis-dienelactone
3-chlorodienelactone
neutral pH
cis-acetylacrylate
maleylacetate
3-chloromaleylacetate
neutral pH
trans-acetylacrylate
3-oxoadipate
TCA cycle
3-hydroxymuconolactone

Fig. 2.

Table S1. Degradation kinetic equations of each monochlorophenol estimated for *A. nidulans* (batch cultivation) and the time (days) to degrade 50% (t_{50}) and 90% (t_{90}) of the initial concentration.

Compound	Best fit non linear equation	R^2	t_{50}	t_{90}
2CP	Y=758.5±22.13*exp(–exp(0.8241±0.08436*(X–6.266±0.1130))) – 0.03340±15.54	0.9888	5.8	7.3
3CP	Y=795.1±33.73*exp(–exp(0.8656±0.1083*(X–3.935±0.1309))) – 2.040±14.24	0.9865	3.5	4.9
4CP	Y=795.3±15.95*exp(–0.3089±0.0164*X) – 33.11±13.02	0.9919	2.2	6.4

Table S2. UHPLC-HRMS data for the identified compounds in each independent culture extract.

Sample	Target Name	Area	RT (min)	Formula	Expected m/z	Measured m/z	Delta m/z (ppm)	Isotopic Pattern Score (%)	Num Isotopes Matched	Adducts
2CP; 2d	2CP sulfate conjugate	3.05E+08	4.12	C6H5ClO4S	206.9524	206.9526	0.85	100	7 of 9	H-Loss** HCO2-Gain
2CP; 2d	3CC sulfate conjugate	4.02E+07	4.38	C6H5ClO5S	222.9473	222.9475	0.74	100	5 of 6	H-Loss** HCO2-Gain
2CP; 2d	chlorobenzoquinone	7.20E+07	4.52	C6H3ClO2	186.9804	186.9808	2.21	100	6 of 6	H-Loss HCO2-Gain**
2CP; 2d	3CC	1.46E+09	4.62	C6H5ClO2	142.9905	142.9904	-0.58	100	6 of 9	H-Loss** HCO2-Gain
2CP; 2d	2CP isomer	9.66E+06	4.90	C6H5ClO	126.9956	126.9957	0.76	94	3 of 4	H-Loss** HCO2-Gain
2CP; 3d	catechol	5.37E+08	3.56	C6H6O2	109.0295	109.0294	-0.77	100	5 of 5	H-Loss** HCO2-Gain
2CP; 3d	2-chloro-*cis*,*trans*-muconate	3.52E+07	3.67	C6H5ClO4	174.9804	174.9805	0.88	100	4 of 5	H-Loss** HCO2-Gain
2CP; 3d	2CP sulfate conjugate	1.28E+09	4.11	C6H5ClO4S	206.9524	206.9523	-0.48	100	7 of 9	H-Loss** HCO2-Gain
2CP; 3d	3CC sulfate conjugate	2.80E+08	4.36	C6H5ClO5S	222.9473	222.9473	-0.01	100	7 of 8	H-Loss** HCO2-Gain
2CP; 3d	chlorobenzoquinone	2.18E+08	4.52	C6H3ClO2	186.9804	186.9808	2.21	100	6 of 6	H-Loss HCO2-Gain**
2CP; 3d	3CC	5.11E+09	4.61	C6H5ClO2	142.9905	142.9905	-0.47	100	9 of 10	H-Loss** HCO2-Gain
2CP; 3d	2CP isomer	5.70E+07	4.90	C6H5ClO	126.9956	126.9957	0.82	100	4 of 4	H-Loss** HCO2-Gain
2CP; 3d	2CP	1.58E+07	5.23	C6H5ClO	126.9956	126.9957	0.64	100	4 of 4	H-Loss** HCO2-Gain

2CP; 8d	protoanemonin	1.96E+08	1.33	C5H4O2	95.0139	95.0140	1.07	100	2 of 3	H-Loss** HCO2-Gain
2CP; 8d	2-chloro-*cis,trans*-muconate	5.35E+08	3.67	C6H5ClO4	174.9804	174.9804	0.45	100	6 of 7	H-Loss** HCO2-Gain
2CP; 8d	2CP sulfate conjugate	8.16E+08	4.08	C6H5ClO4S	206.9524	206.9524	-0.11	100	7 of 9	H-Loss** HCO2-Gain
2CP; 8d	3CC sulfate conjugate	1.20E+09	4.34	C6H5ClO5S	222.9473	222.9474	0.46	100	9 of 10	H-Loss** HCO2-Gain
2CP; 8d	chlorobenzoquinone	1.85E+08	4.52	C6H3ClO2	186.9804	186.9805	0.83	100	6 of 6	H-Loss HCO2-Gain**
2CP; 8d	3CC	3.19E+09	4.62	C6H5ClO2	142.9905	142.9903	-1.64	100	9 of 11	H-Loss** HCO2-Gain*
2CP; 8d	2CP isomer	1.30E+08	4.91	C6H5ClO	126.9956	126.9956	-0.5	100	5 of 5	H-Loss** HCO2-Gain
3CP; 2d	3-hydroxymuconolactone	6.14E+07	1.31	C6H6O5	157.0142	157.0143	0.61	100	2 of 3	H-Loss** HCO2-Gain
3CP; 2d	3-chlorodienelactone	2.08E+09	2.10	C6H3ClO4	172.9647	172.9648	0.33	100	6 of 8	H-Loss** HCO2-Gain
3CP; 2d	chloromuconolactone	1.58E+08	2.29	C6H5ClO4	174.9804	174.9804	0.45	100	5 of 6	H-Loss** HCO2-Gain
3CP; 2d	2-chloro-*cis,cis*-muconate	9.56E+06	3.33	C6H5ClO4	174.9804	174.9808	2.72	100	3 of 3	H-Loss** HCO2-Gain
3CP; 2d	catechol	2.98E+08	3.56	C6H6O2	109.0295	109.0295	-0.07	100	3 of 4	H-Loss** HCO2-Gain
3CP; 2d	2-chloro-*cis,trans*-muconate	6.68E+08	3.68	C6H5ClO4	174.9804	174.9805	0.71	100	6 of 7	H-Loss** HCO2-Gain
3CP; 2d	3-chloro-*cis,trans*-muconate	6.56E+08	4.30	C6H5ClO4	174.9804	174.9804	0.19	100	6 of 6	H-Loss** HCO2-Gain
3CP; 2d	3CC sulfate conjugate	2.25E+08	4.36	C6H5ClO5S	222.9473	222.9473	-0.42	100	7 of 9	H-Loss** HCO2-Gain
3CP; 2d	4CC sulfate conjugate	4.58E+08	4.43	C6H5ClO5S	222.9473	222.9473	-0.29	100	7 of 9	H-Loss** HCO2-Gain
3CP; 2d	3CC	3.98E+09	4.62	C6H5ClO2	142.9905	142.9904	-1.11	100	8 of 10	H-Loss**

3CP; 2d	4CC	2.49E+10	4.76	C6H5ClO2	142.9905	142.9903	-1.54	100	10 of 12	HCO2-Gain H-Loss** HCO2-Gain
3CP; 2d	unknown conjugate	6.60E+08	5.09	C10H11ClO4	229.0273	229.0272	-0.52	100	4 of 9	H-Loss** HCO2-Gain*
3CP; 2d	3CP	7.56E+09	5.44	C6H5ClO	126.9956	126.9956	0.22	100	8 of 11	H-Loss** HCO2-Gain*
3CP; 2d	unknown conjugate	1.36E+08	5.54	C9H9ClO3	199.0167	199.0170	1.50	100		H-Loss** HCO2-Gain
3CP; 3d	3-hydroxymuconolactone	1.08E+08	1.37	C6H6O5	157.0142	157.0143	0.61	100	2 of 3	H-Loss** HCO2-Gain
3CP; 3d	*trans*-acetylacrylate	2.85E+08	1.90	C5H6O3	113.0244	113.0246	1.25	100	3 of 3	H-Loss** HCO2-Gain
3CP; 3d	3-chlorodienelactone	6.32E+09	2.01	C6H3ClO4	172.9647	172.9648	0.77	100	7 of 8	H-Loss** HCO2-Gain
3CP; 3d	chloromuconolactone	1.97E+08	2.32	C6H5ClO4	174.9804	174.9805	0.97	100	6 of 6	H-Loss** HCO2-Gain
3CP; 3d	2-chloro-*cis,cis*-muconate	6.74E+07	3.31	C6H5ClO4	174.9804	174.9809	2.89	91	4 of 5	H-Loss** HCO2-Gain
3CP; 3d	dienelactone	7.49E+08	3.35	C6H4O4	139.0037	139.0036	-0.25	100	4 of 5	H-Loss** HCO2-Gain
3CP; 3d	catechol	4.76E+08	3.56	C6H6O2	109.0295	109.0295	-0.35	100	4 of 5	H-Loss** HCO2-Gain*
3CP; 3d	2-chloro-*cis,trans*-muconate	1.58E+09	3.70	C6H5ClO4	174.9804	174.9805	1.06	100	6 of 7	H-Loss** HCO2-Gain
3CP; 3d	chloromuconate	1.31E+07	4.04	C6H5ClO4	174.9804	174.9805	0.71	100	3 of 3	H-Loss** HCO2-Gain
3CP; 3d	3-chloro-*cis,trans*-muconate	8.26E+08	4.29	C6H5ClO4	174.9804	174.9805	0.97	100	6 of 7	H-Loss** HCO2-Gain
3CP; 3d	chlorotrihydroxybenzene	6.23E+07	4.11	C6H5ClO3	158.9854	158.9856	0.75	100	4 of 5	H-Loss** HCO2-Gain
3CP; 3d	3CC sulfate conjugate	1.90E+09	4.33	C6H5ClO5S	222.9473	222.9474	0.33	100	9 of 10	H-Loss** HCO2-Gain

3CP; 3d	4CC sulfate conjugate	8.57E+08	4.40	C6H5ClO5S	222.9473	222.9475	0.74	100	9 of 10	H-Loss** HCO2-Gain
3CP; 3d	3CC	5.28E+10	4.62	C6H5ClO2	142.9905	142.9904	-0.58	100	10 of 13	H-Loss** HCO2-Gain
3CP; 3d	4CC	7.92E+10	4.75	C6H5ClO2	142.9905	142.9904	-0.58	100	10 of 13	H-Loss** HCO2-Gain
3CP; 3d	3CP sulfate conjugate	9.97E+07	4.07	C6H5ClO4S	206.9524	206.9526	0.92	100	5 of 7	H-Loss** HCO2-Gain
3CP; 3d	unknown conjugate		5.10	C10H11ClO4	229.0273	229.0273	-0.26	100	6 of 6	H-Loss** HCO2-Gain
3CP; 3d	unknown conjugate	6.74E+07	5.54	C9H9ClO3	199.0167	199.0170	1.42	100	6 of 9	H-Loss** HCO2-Gain
3CP; 3d	3CP	3.04E+09	5.45	C6H5ClO	126.9956	126.9956	-0.2	100	8 of 9	H-Loss** HCO2-Gain*
3CP; 8d	3-hydroxymuconolactone	5.59E+07	1.43	C6H6O5	157.0142	157.0142	-0.36	100	3 of 3	H-Loss** HCO2-Gain
3CP; 8d	*trans*-acetylacrylate	1.59E+09	1.83	C5H6O3	113.0244	113.0244	-0.23	100	5 of 6	H-Loss** HCO2-Gain
3CP; 8d	3-chlorodienelactone	5.89E+09	2.04	C6H3ClO4	172.9647	172.9646	-0.37	100	7 of 8	H-Loss** HCO2-Gain
3CP; 8d	2-chloro-*cis*,*cis*-muconate	1.07E+08	3.33	C6H5ClO4	174.9804	174.9810	3.67	100	5 of 6	H-Loss** HCO2-Gain
3CP; 8d	2-chloro-*cis*,*trans*-muconate	2.40E+09	3.69	C6H5ClO4	174.9804	174.9806	1.15	100	6 of 8	H-Loss** HCO2-Gain
3CP; 8d	chloromuconate	2.87E+07	4.04	C6H5ClO4	174.9804	174.9805	1.06	100	4 of 5	H-Loss** HCO2-Gain
3CP; 8d	3-chloro-*cis*,*trans*-muconate	6.84E+08	4.30	C6H5ClO4	174.9804	174.9805	1.06	100	6 of 7	H-Loss** HCO2-Gain*
3CP; 8d	4CC sulfate conjugate	3.36E+09	4.39	C6H5ClO5S	222.9473	222.9475	0.88	100	10 of 11	H-Loss** HCO2-Gain
3CP; 8d	chlorobenzoquinone	6.14E+07	4.61	C6H3ClO2	186.9804	186.9808	2.13	100	4 of 6	H-Loss HCO2-Gain**
3CP; 8d	3CC	4.45E+07	4.62	C6H5ClO2	142.9905	142.9905	0.06	100	5 of 7	H-Loss**

3CP; 8d	4CC	2.41E+08	4.76	C6H5ClO2	142.9905	142.9904	-1.11	100	5 of 7	HCO2-Gain* H-Loss**
3CP; 8d	unknown conjugate		5.10	C10H11ClO4	229.0273	229.0273	-0.13	100	6 of 9	HCO2-Gain H-Loss**
3CP; 8d	unknown conjugate	7.06E+07	5.54	C9H9ClO3	199.0167	199.0171	2.11	100		HCO2-Gain H-Loss**
4CP; 2d	3-hydroxymuconolactone	6.46E+07	1.44	C6H6O5	157.0142	157.0143	0.42	100	2 of 3	HCO2-Gain H-Loss**
4CP; 2d	3-chlorodienelactone	3.88E+08	2.16	C6H3ClO4	172.9647	172.9647	0.16	100	7 of 7	HCO2-Gain H-Loss**
4CP; 2d	dienelactone	5.00E+08	3.35	C6H4O4	139.0037	139.0038	0.52	100	5 of 6	HCO2-Gain H-Loss**
4CP; 2d	3-chloro-*cis,trans*-muconate	3.51E+08	4.29	C6H5ClO4	174.9804	174.9804	0.27	100	6 of 7	HCO2-Gain* H-Loss**
4CP; 2d	chlorotrihydroxybenzene	6.68E+07	4.11	C6H5ClO3	158.9854	158.9853	-1.07	100	5 of 7	HCO2-Gain H-Loss**
4CP; 2d	4CC sulfate conjugate	4.18E+08	4.44	C6H5ClO5S	222.9473	222.9474	0.05	100	7 of 9	HCO2-Gain H-Loss**
4CP; 2d	4CC	2.36E+10	4.75	C6H5ClO2	142.9905	142.9903	-1.32	100	11 of 11	HCO2-Gain H-Loss**
4CP; 2d	unknown conjugate		5.09	C10H11ClO4	229.0273	229.0270	-1.53	100	4 of 6	HCO2-Gain H-Loss**
4CP; 2d	4CP	5.65E+09	5.38	C6H5ClO	126.9956	126.9955	-1.04	100	9 of 11	HCO2-Gain* H-Loss**
4CP; 2d	unknown conjugate	3.10E+07	5.53	C9H9ClO3	199.0167	199.0169	0.73	100		HCO2-Gain* H-Loss**
4CP; 3d	3-hydroxymuconolactone	1.10E+08	1.43	C6H6O5	157.0142	157.0143	0.42	100	2 of 3	HCO2-Gain H-Loss**
4CP; 3d	3-chlorodienelactone	2.91E+09	2.12	C6H3ClO4	172.9647	172.9646	-0.37	100	7 of 8	HCO2-Gain H-Loss**
4CP; 3d	dienelactone	1.23E+09	3.35	C6H4O4	139.0037	139.0038	0.52	100	5 of 6	HCO2-Gain H-Loss**
										HCO2-Gain

4CP; 3d	3-chloro-*cis*,*trans*-muconate	9.40E+08	4.29	C6H5ClO4	174.9804	174.9806	1.15	100	6 of 7	H-Loss** HCO2-Gain
4CP; 3d	chlorotrihydroxybenzene	1.52E+07	4.11	C6H5ClO3	158.9854	158.9857	1.43	100	3 of 4	H-Loss** HCO2-Gain
4CP; 3d	4CC sulfate conjugate	2.47E+09	4.42	C6H5ClO5S	222.9473	222.9475	0.6	100	8 of 10	H-Loss** HCO2-Gain
4CP; 3d	4CC	5.91E+10	4.76	C6H5ClO2	142.9905	142.9904	-0.79	100	10 of 12	H-Loss** HCO2-Gain
4CP; 3d	4CP sulfate conjugate	1.89E+07	4.19	C6H5ClO4S	206.9524	206.9527	1.51	100	5 of 6	H-Loss** HCO2-Gain
4CP; 3d	unknown conjugate		5.09	C10H11ClO4	229.0273	229.0271	-1.05	100	4 of 8	H-Loss** HCO2-Gain
4CP; 3d	4CP	2.94E+09	5.38	C6H5ClO	126.9956	126.9956	-0.14	100	9 of 11	H-Loss** HCO2-Gain*
4CP; 3d	unknown conjugate	8.66E+07	5.53	C9H9ClO3	199.0167	199.0173	2.57	100		H-Loss** HCO2-Gain
4CP; 8d	3-hydroxymuconolactone	3.72E+08	1.43	C6H6O5	157.0142	157.0141	-1.04	100	3 of 3	H-Loss** HCO2-Gain
4CP; 8d	*trans*-acetylacrylate	5.66E+07	1.76	C5H6O3	159.0299	159.0298	-0.51	100	3 of 3	H-Loss HCO2-Gain**
4CP; 8d	3-chlorodienelactone	7.37E+09	2.02	C6H3ClO4	172.9647	172.9646	-0.64	100	7 of 8	H-Loss** HCO2-Gain
4CP; 8d	dienelactone	2.65E+09	3.34	C6H4O4	139.0037	139.0037	0.08	100	4 of 5	H-Loss** HCO2-Gain*
4CP; 8d	3-chloro-*cis*,*trans*-muconate	2.12E+09	4.30	C6H5ClO4	174.9804	174.9804	0.45	100	7 of 8	H-Loss** HCO2-Gain
4CP; 8d	4CC sulfate conjugate	2.80E+09	4.36	C6H5ClO5S	222.9473	222.9475	0.53	100	9 of 11	H-Loss** HCO2-Gain
4CP; 8d	chlorobenzoquinone	2.37E+07	4.62	C6H3ClO2	186.9804	186.9807	1.81	100	4 of 5	H-Loss HCO2-Gain**
4CP; 8d	4CC	1.86E+08	4.76	C6H5ClO2	142.9905	142.9903	-1.32	100	5 of 7	H-Loss** HCO2-Gain
4CP; 8d	4CP sulfate conjugate	3.62E+07	3.99	C6H5ClO4S	206.9524	206.9525	0.33	100	4 of 6	H-Loss**

4CP; 8d	unknown conjugate	5.09	C10H11ClO4	229.0273	229.0272	-0.66	100	4 of 9	HCO2-Gain H-Loss** HCO2-Gain

Table S3. Degradation intermediates detected upon incubation of 4-monochlorocatechol with *A. nidulans* mycelial protein crude extract. The reaction media was analysed by UPLC and, upon its extraction, also by UHPLC-HRMS. For each compound the elemental chemical formula, the retention time ($RT_{LC\text{-}HRMS}$), the expected ion mass, the measured ion mass in negative mode, error and peak area are indicated.

Compound	**Chemical formula**	**Expected ion mass**	**Measured ion mass**	**Error**	**$RT_{LC\text{-}HRMS}$ (min)**	**Area**
chloromaleylacetate	$C_6H_5ClO_5$	190.9753	190.9755	1.39	1.32	1.8E+07
3-hydroxymuconolactone	$C_6H_6O_5$	157.0142	157.0142	-0.36	1.41	4.7E+09
trans-acetylacrylate	$C_5H_6O_3$	113.0244	113.0244	-0.50	1.87	2.0E+08
3-chloromuconolactone [a]	$C_6H_5ClO_4$	174.9804	174.9804	0.27	2.91	3.8E+07
cis-dienelactone [a]	$C_6H_4O_4$	139.0037	139.0036	-0.25	3.35	1.2E+09
3-chloro-*cis*,*trans*-muconate [a]	$C_6H_5ClO_4$	174.9804	174.9805	0.71	4.29	7.8E+09
4CC	$C_6H_5ClO_2$	142.9905	142.9904	-0.68	4.75	7.3E+08

[a] also detected in the organic extracts by liquid chromatography

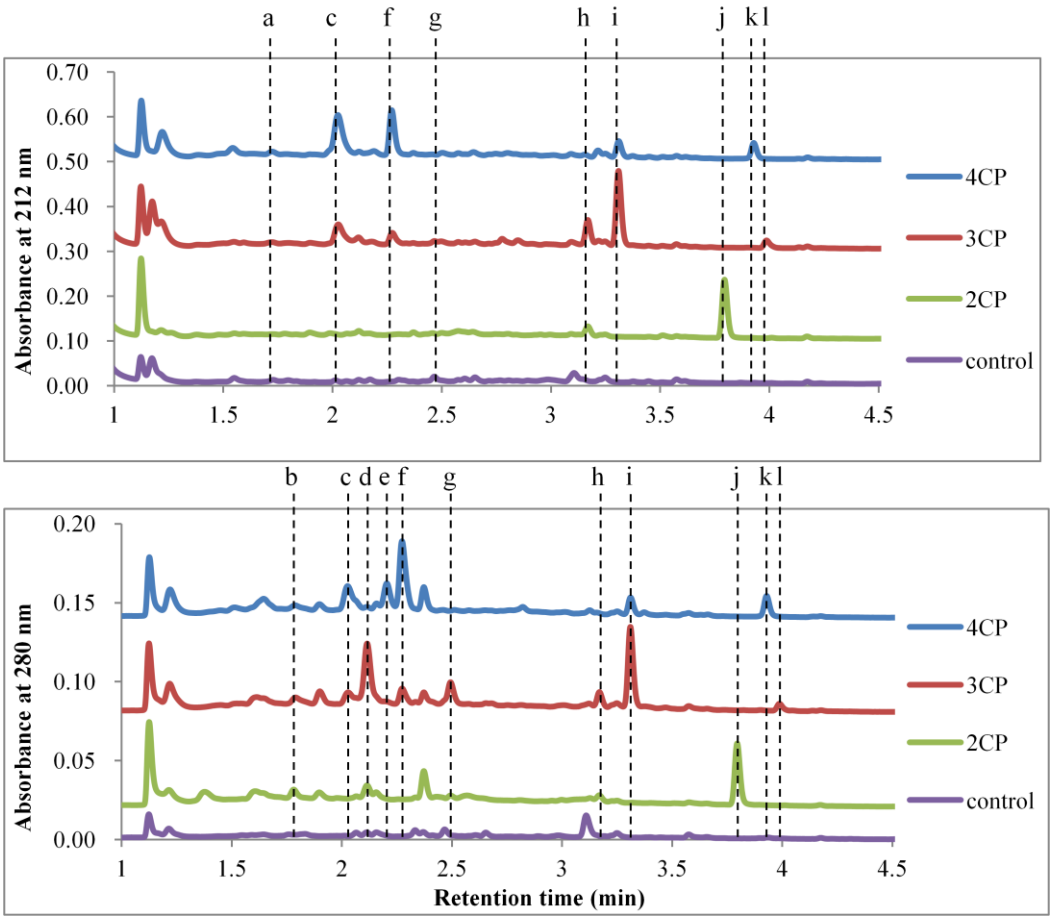

Fig. S1. Chromatographic profiles of *Aspergillus nidulans* culture media filtrate at the last time point of monochlorophenols detection, *i.e.*, five days for 3CP (j) and seven days for 2CP (l), 4CP (k) and biotic control. At these time points the degradation intermediates detected, included, *trans*-acetylacrylate (a), *cis*,*cis*-muconate (b), X211 (c), X280a (d), *cis*-dienelactone (e), 3-chloro-*cis*,*cis*-muconate (f), X280b (g), 3CC (h) and 4CC (i).

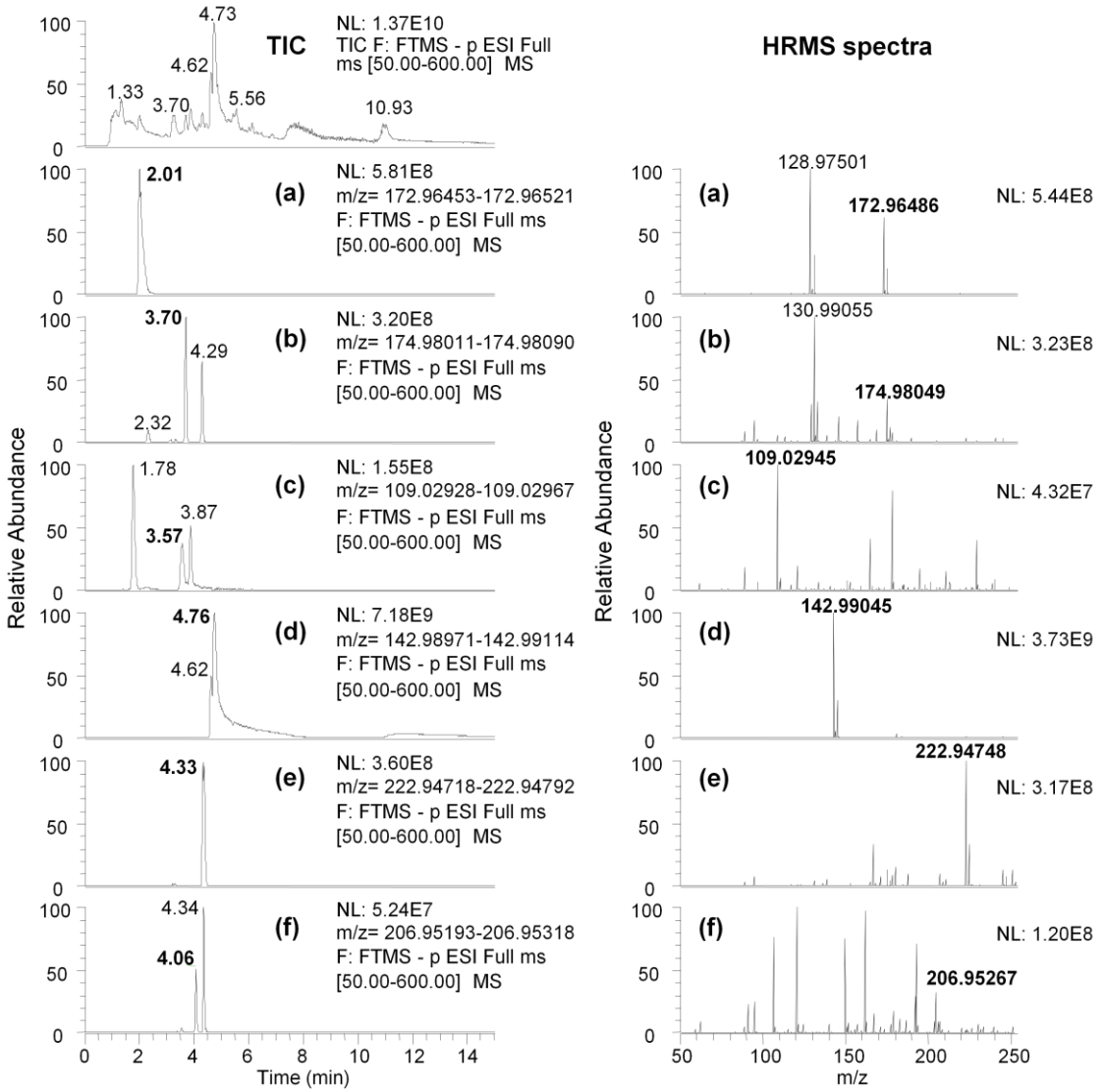

Fig. S2. The total ion chromatogram (TIC), the extracted ion chromatogram and HRMS spectra for peaks corresponding to 3-chlorodienelactone (a: RT=2.01 min), 2- and 3-chloro-*cis*,*trans*-muconate (b: RT=3.70 and 4.29 min), catechol (c: RT=3.57 min), 3CC and 4CC (d: RT=4.62 and 4.76 min), 3CC sulfate conjugate (e: RT=4.33 min) and 3CP sulfate conjugate (f: RT=4.06 min), of a representative sample (3 days, 3CP culture) is shown. HRMS spectra are only presented for the compounds with RT in bold.

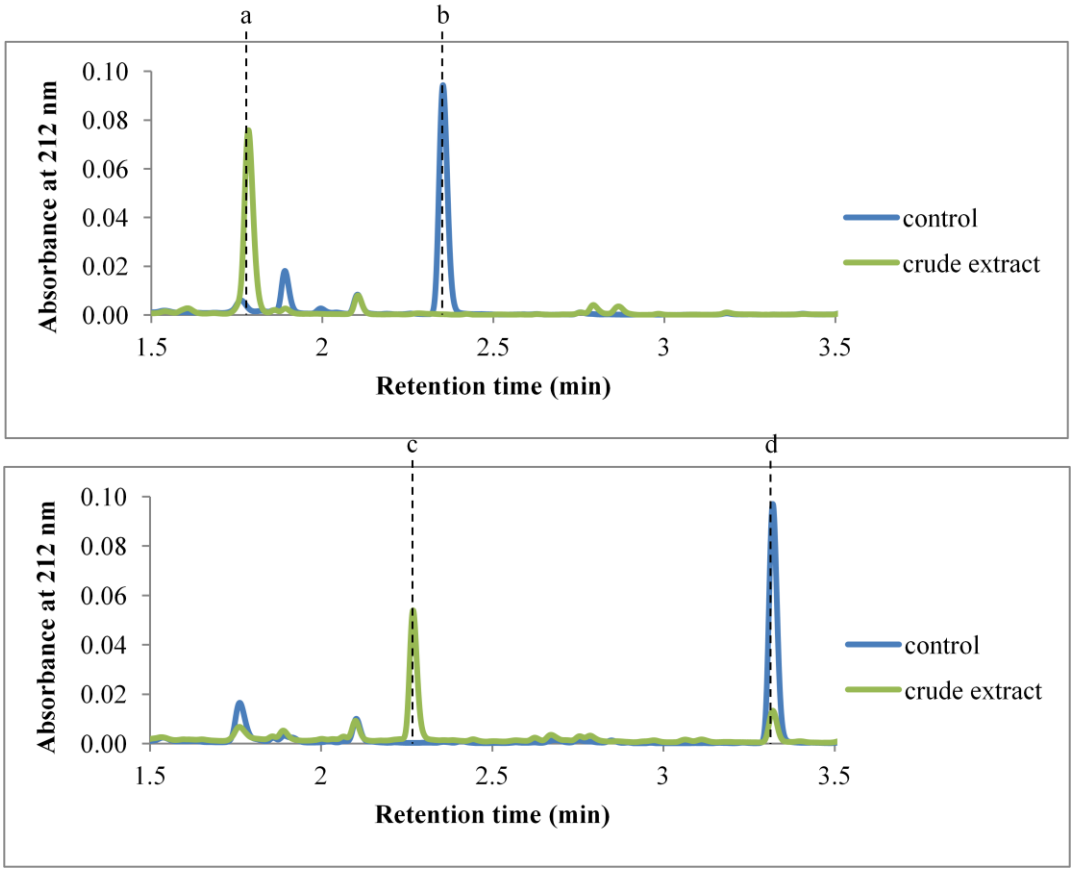

Fig. S3. Chromatographic profiles of the transformation of catechols by *Aspergillus nidulans* mycelial protein crude extract with dioxygenase activity: catechol (b) yielded *cis,cis*-muconate (a) and 4CC (d) yielded 3-chloro-*cis,cis*-muconate (c). The products were not detected in abiotic conditions as shown by analysis of a boiled crude extract (control).