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Mapping the structural requirements of inducers and substrates for decarboxylation of weak acid preservatives by the food spoilage mould *Aspergillus niger*

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ABSTRACT

Moulds are able to cause spoilage in preserved foods through degradation of the preservatives using the Pad-decarboxylation system. This causes, for example, decarboxylation of the preservative sorbic acid to 1,3-pentadiene, a volatile compound with a kerosene-like odour. Neither the natural role of this system nor the range of potential substrates has yet been reported. The Pad-decarboxylation system, encoded by a gene cluster in germinating spores of the mould *Aspergillus niger*, involves activity by two decarboxylases, PadA1 and OhbA1, and a regulator, SdrA, acting pleiotropically on sorbic acid and cinnamic acid. The structural features of compounds important for the induction of Pad-decarboxylation at both transcriptional and functionality levels were investigated by rtPCR and GCMS. Sorbic and cinnamic acids served as transcriptional inducers but ferulic, coumaric and hexanoic acids did not. 2,3,4,5,6-Pentafluorocinnamic acid was a substrate for the enzyme but had no inducer function; it was used to distinguish induction and competence for decarboxylation in combination with the analogue chemicals. The structural requirements for the substrates of the Pad-decarboxylation system were probed using a variety of sorbic and cinnamic acid analogues. High decarboxylation activity, ~100% conversion of 1 mM substrates, required a mono-carboxylic acid with an alkenyl double bond in the *trans* (*E*)-configuration at position C2, further unsaturation at C4, and an overall molecular length between 6.5 Å and 9 Å. Polar groups on the phenyl ring of cinnamic acid abolished activity (no conversion). Furthermore, several compounds were shown to block Pad-decarboxylation. These compounds, primarily aldehyde analogues of active substrates, may serve to reduce food spoilage by moulds such as *A. niger*. The possible ecological role of Pad-decarboxylation of spore self-inhibitors is unlikely and the most probable role for Pad-decarboxylation is to remove cinnamic acid-type inhibitors from plant material and allow uninhibited germination and growth of mould spores.

1. Introduction

Filamentous fungi form non-sexual spores (conidia) in large numbers for airborne dissemination and propagation of the species. On a suitable substrate, the spore will germinate, extend a germ tube, and form a spreading hyphal mass. The spore may be faced with a variety of antimicrobial compounds and the ability of fungal spores to overcome such inhibitory compounds is of major benefit to the survival and propagation of the species. Such antimicrobial factors include hydroxycinnamic acids and essential oils of plant origin, antimicrobial agents from competing micro-organisms (Lachance, 1989) or from the spores themselves (Garrett and Robinson, 1969). Many inhibitory compounds, such as nonanoic acid (Breeuwer et al., 1997) or 1-octen-3-ol (Chitarra et al., 2004) have been reported in fungal spores, acting as self-inhibitors of spore germination, whose function appears to be prevention of spore germination until the spores are sufficiently dispersed. Germinating spores of the filamentous fungus *Aspergillus niger* efficiently decarboxylate sorbic and cinnamic acids but the ecological advantage of so doing is unknown.

In the food industry, sorbic acid (2,4-hexadienoic acid) is permitted as a preservative (Anon, 1995) of low-pH, sugar-containing products, while cinnamic acid (3-phenyl-2-propenoic acid) is permitted within

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the EU as a food-flavouring agent (Burdock, 2002) but is a powerful antimicrobial agent, patented for use in beverages (Stratford and Anslow, 1997). Some spoilage moulds, such as *A. niger*, are able to decarboxylate sorbic acid to the volatile and less toxic 1,3-pentadiene and thereby exhibit resistance to this compound (Plumridge et al., 2004). Fungal decarboxylation of sorbic acid not only potentiates spoilage through visible mould outgrowth and generation of the kerosene-like odour of 1,3-pentadiene, but also permits growth of other, sorbic-acid-sensitive, microbes. Decarboxylation of sorbic acid to 1,3-pentadiene has been demonstrated in several mould species, including *Trichoderma* and *Penicillium* spp. and in a few yeast species (Marth et al., 1966; Kurogochi et al., 1975; Kinderlerler and Hutton, 1990; Casas et al., 1999, 2004; Pinches and Apps, 2007).

The activity of a cinnamic acid decarboxylase, encoded by the gene *padA1* (*PAD1* in the yeast *Saccharomyces cerevisiae*) (Clausen et al., 1994) is responsible for the decarboxylation of both sorbic and cinnamic acids in germinating spores of *A. niger* (Plumridge et al., 2008). Alternative names for cinnamic acid include phenylacrylic acid (Clausen et al., 1994) but more correctly, 3-phenyl-(*E*)-2-propenoic acid or *tert*-β-phenylacrylic acid (Burdock, 2002). Disruption of the *padA1* gene resulted in 50% lower concentrations of sorbic acid to prevent conidial outgrowth. In contrast, in the yeast *S. cerevisiae*, *PAD1* activity is slight and gene disruption did not alter resistance to sorbic acid (Stratford et al., 2007) demonstrating that Pad activity did not contribute to preservative resistance in that yeast.

The view that decarboxylase activity depended solely on the induction of *pad* genes was shown to be an over-simplification by the discovery (Plumridge et al., 2010) that the decarboxylation process in *A. niger* also requires activity of a putative 2-hydroxybenzoic acid decarboxylase, encoded by *ohbA1* (3-octaprenyl-2-hydroxybenzoic acid decarboxylase) and a putative transcription factor encoded by *sdrA* (sorbic acid decarboxylase regulator). These three genes, *padA1*, *ohbA1* and *sdrA*, form a cluster on chromosome 6 in *A. niger*. Two other homologous clusters, *padA2*/*ohbA2* and *padA3*/*ohbA3*, are present at other loci in the *A. niger* genome but are not expressed in the presence of sorbic acid. Further bioinformatic analysis showed that this clustering was highly conserved in several *Aspergillus* species and also, with the exception of a homologue of *sdrA*, in the yeast *S. cerevisiae* (Mukai et al., 2010). This conserved synteny indicates a clustering of metabolic function and regulation, although the role of the PadA1 and OhbA1 proteins, together or in sequence in the decarboxylation process (referred to subsequently as the Pad-decarboxylation system), remains to be revealed.

The objectives of this study were to identify the structural features of chemicals that transcriptionally induce the Pad-decarboxylation system in developing conidia of *A. niger* and to define the structural features that determine the substrate acceptability by the decarboxylase system. The (unknown) complexity of the Pad-decarboxylation system mitigates against the use of X-ray crystallography although there are crystal structures of purified Pad-decarboxylases from *Escherichia coli* (Protein Data Bank, PDB, entry 1sbz; Rangarajan et al., 2004) and *Aquifex aeolicus* (PDB entry 2ejb). A wide variety of analogues of sorbic acid and cinnamic acid were used to probe the active site of the decarboxylase system. Such an approach is analogous to the fragment-based methods of active site probing (Hann et al., 2001; Ciulli and Abell, 2007) used in combination with dynamic combinatorial chemistry and high-throughput screening to develop small molecule drugs in the medical field. In such studies, the active sites of key enzymes in pathogenic bacteria were probed using a variety of single low-molecular weight chemical fragments, <250 Da (Murray and Verdonk, 2002; Ciulli and Abell, 2007). Fragment binding enabled identification of key protein residues and design of novel antimicrobiological drugs (Ciulli et al., 2008; Olivier and Imperiali, 2008; Belin et al., 2009; Hung et al., 2009; Scott et al., 2009; Larkin et al., 2010). The possible ecological role for the Pad-decarboxylation system is also discussed.

2. Materials and methods

2.1. A. niger strains

Two strains of *A. niger* were used in these studies. Decarboxylation occurred in strain N402 (Bos et al., 1988) while strain AXP6-2.21a (Δ*padA1*) completely lacked the ability to decarboxylate sorbic or cinnamic acids (Plumridge et al., 2008).

2.2. Media and growth conditions

A. niger strains were grown on Potato Dextrose Agar (PDA, Oxoid Ltd., Basingstoke, Hampshire, UK, pH 5.6 ± 0.2) slopes or plates for 5 days at 28 °C to develop mature conidia. Conidia were harvested by washing with 0.1% w/v Tween 80 in deionized water and were counted using a haemocytometer.

2.3. Decarboxylation testing in intact spores

Weak-acid decarboxylation was tested in YEPD medium adjusted to pH 4.0 with 10 M HCl (glucose 2% w/v, peptone 2% w/v, yeast extract 1% w/v) containing acids/substrates at 1 mM. 10 ml aliquots in 28 ml McCartney bottles were inoculated with conidia at 10^7/ml (t = 0) and incubated at 28 °C for 10 h. Controls showed no germination of conidia or initiation of sorbic acid decarboxylation in 0.1% Tween 80. Volatiles in headspace samples were detected and quantified by GCMS as described previously (Stratford et al., 2007; Plumridge et al., 2008). Quantification of decarboxylation of sorbic acid and cinnamic acid was determined using 1,3-pentadiene and styrene standards respectively. Standards were accurately prepared in YEPD at 1 mM and incubated alongside experimental cultures for 10 h. Tests showed that equilibrated standards of 1,3-pentadiene and styrene gave a linear increase in headspace GCMS peak area over the range 0–3 mM. Decarboxylation standards derived from many other substrates were not available, so conclusions drawn from the results were kept semi-quantitative; absent, present at low level, or high level (peak areas 0, <4000, >22,000). In control tests to determine volatiles generated by *A. niger* conidia in the absence of exogenous substrates, using inocula at 10^7/ml–10^9/ml with a 10 ml sample volume, no compounds were found capable of being products of the Pad-decarboxylation system.

2.4. Chemicals

All of the compounds tested as decarboxylation substrates were obtained from Sigma-Aldrich or Alfa Aesar, unless stated otherwise. Longer-chain analogues of sorbic acid were prepared by oxidation of their corresponding, commercially-available aldehydes, obtained from Sigma (Flavours and Fragrances). The 2,4-dienals were oxidised to the corresponding carboxylic acids using a protocol employed for the oxidation of 4-oxo-2-enals (Kobayashi et al., 1998) and the configurationally-pure *trans* (*E*)-2, *trans* (*E*)-4 isomers were purified by chromatography and characterised by NMR spectroscopy.

2.5. Decarboxylation in cell-free extracts

Three 100 ml aliquots of YEPD pH 4.0 in 500 ml conical flasks were inoculated with *A. niger* conidia at 10^8/ml. 2 mM sorbic acid was added to induce decarboxylation activity. Flasks were shaken at 140 rpm for 6 h at 28 °C. Germinating conidia were obtained by centrifugation (5000 × *g*, 5 min), washed twice and resuspended in 50 mM Tris pH 7.5 at 5×10^9/ml and snap frozen in liquid nitrogen. Suspensions of germinating conidia (3 ml aliquots) were then thawed, added to glass balls (2 g, 0.5 mm) and broken by vortexing for 10 min. Tubes were re-chilled every 2 min in liquid nitrogen. Microscopic examination confirmed that >99% conidia were broken.

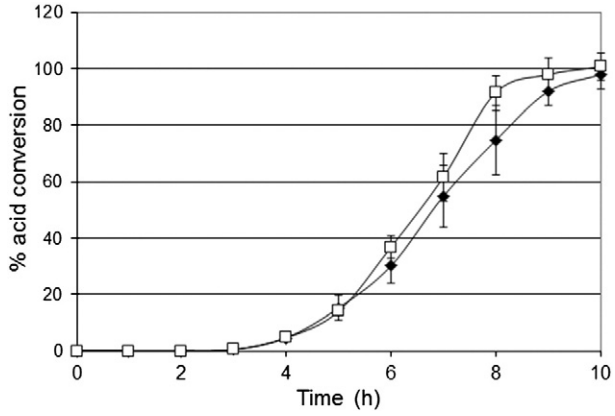


Fig. 1. Time course of the conversion of 1 mM sorbic acid into 1,3-pentadiene (closed symbols) and 1 mM cinnamic acid into styrene (open symbols) by germinating conidia of *Aspergillus niger*. Volatiles were detected by GCMS of headspace samples. 1,3-Pentadiene standards, prepared at 1 mM and equilibrated for 10 h gave a mean peak area of 41,876 and 1 mM styrene gave a peak area of 48,202.

Extracts were combined and centrifuged to remove glass balls, whole cells and cell debris. The supernatant was made up to 30 ml with extraction buffer (50 mM Tris pH 7.5, 5% w/v glycerol) and dispensed at 1 ml into 28 ml McCartney bottles. Decarboxylation substrates were added to cell-free extracts at 1 mM. Bottles were sealed and incubated, shaken at 120 rpm for 24 h at 28 °C. To maximise sensitivity without overloading GCMS peaks, headspace samples were increased to 10 ml.

2.6. Testing inducer and/or substrate function for decarboxylation

2,3,4,5,6-Pentafluorocinnamic acid was employed, together with a wide range of other compounds, to assess their functionality as inducers of the Pad-decarboxylation system. 2,3,4,5,6-Pentafluorocinnamic acid is an analogue of cinnamic acid and was shown to be a substrate of Pad-decarboxylation but not an inducer of the system. The supporting data for the use of 2,3,4,5,6-pentafluorocinnamic acid in this way, and the precise conditions, are described in Results section.

2.7. RNA extraction and qRT-PCR

A. niger conidia (10^4/ml) were germinated for 6 h at 28 °C, in conical flasks shaken at 150 rpm. Conidia were recovered by filtration, and frozen in 0.5 ml RNA extraction buffer (0.6 M NaCl, 0.2 M sodium acetate, 0.1 M EDTA, 4% w/v SDS). Frozen conidia were mixed with glass beads and broken in a Sartorius dismembranator (4 min, 200 rpm), followed by Trizol extraction and isopropanol precipitation. Samples were DNase treated and purified using RNeasy columns (Qiagen GmbH, Hilden, Germany).

qRT-PCR amplifications were carried out using the Applied Biosystems 7500 Fast Real-Time PCR system. Total RNA SuperScript™ III reverse transcriptase (Invitrogen) was used to prepare cDNA. The PCR reaction mixture (10 µl) contained 25 ng cDNA, specific primer sets (175 nM final concentration), and FAST SYBR-Green Master Mix (Applied Biosystems). PCRs were carried out for 40 cycles; denaturation at 95 °C for 15 s, annealing at 67 °C for 30 s, and extension at 60 °C for 60 s using primers for padA1 gene: padA1-up 5′-TGTAGTAGCCATGACGGGTGCCACTG-3′, *padA1*-down 5′-TGAGATCGTCGCAAAAGCCCGAG-3′ and for *ohbA1* gene: ohbA1-up 5′-TCACCTAGCCCTCCCACCTA-3′, ohbA1-down 5′-TCCAGGGA GGATCCCGTCAT-3′.

All experiments were independently conducted 3 times. The specificity of primer sets used for qRT-PCR amplification was evaluated by melting curve analysis. The standard curve method was used for data evaluation (Liu et al., 2009).

3. Results

3.1. Time-course of induction of decarboxylation

The decarboxylation system used by germinating conidia of *A. niger* N402 to convert sorbic acid into *trans* (*E*)-1,3-pentadiene requires induction at the transcriptional level. The time course of the development of decarboxylation activity is recorded in Fig. 1. This shows that following addition of either sorbic acid or cinnamic acid to the germinating conidia, no decarboxylation was detectable initially, and was barely detectable at 3 h, but it increased thereafter. At 6 h ~30% of the acids had been removed by decarboxylation, leaving 70% to continue to act as an inducer. Close to 100% decarboxylation of sorbic acid or cinnamic acid was achieved in 10 h.

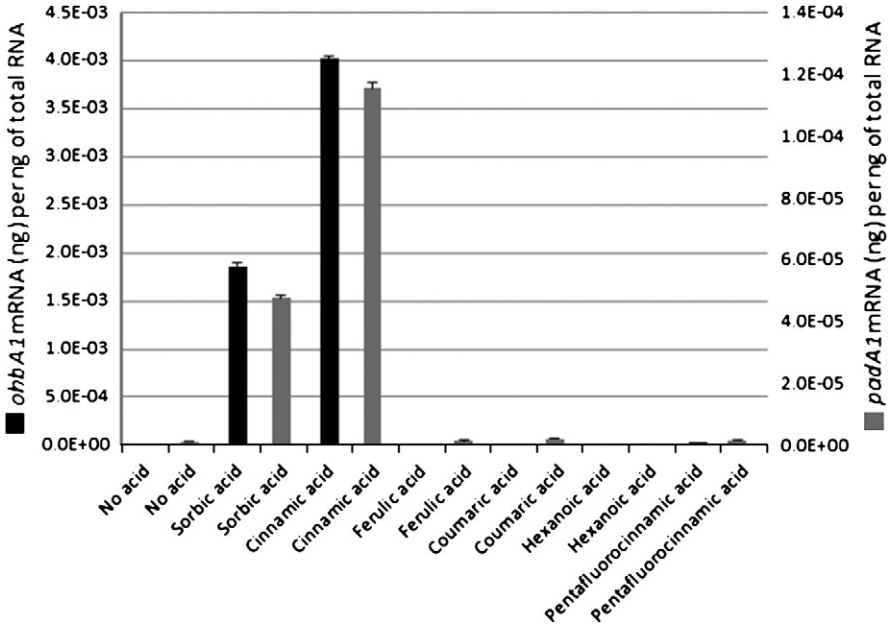


Fig. 2. Expression levels of *padA1* (Fig. 2a) and *ohbA1* (Fig. 2b) genes in germinating *A. niger* conidia in the presence of various weak acids. Data shown represent mean *padA1* and *ohbA1* mRNA amounts extracted at 6 h in conidial germination. Variability in expression levels across three independent experiments is shown by error bars.

No decarboxylation activity was found in 6 h cell-free extracts of germinating conidia without prior incubation with either sorbic acid or cinnamic acid, confirming that the decarboxylation system required induction (data not shown). In cell-free extracts taken at 6 h, decarboxylation activity induced by sorbic acid was active against cinnamic acid and vice versa. In *A. niger* strain AXP6-2.21a (Δ*padA1*), no decarboxylation activity was induced by either sorbic acid or cinnamic acid, and no decarboxylation activity was detected in cell-free extracts. From these data, it was concluded that both sorbic acid and cinnamic acid acted as inducers for the Pad-decarboxylation system, and that both acids function as substrates for that system which we know from the previous studies (Plumridge et al., 2010) requires both *padA1* and *obhA1*.

Confirmation that induction at the transcriptional levels required either sorbic acid or cinnamic acid was shown using qRT-PCR (Fig. 2). Expression of *padA1* and *ohbA1* genes occurred at a low level in the process of germination without prior incubation with sorbic or cinnamic acid. Upon induction, the expression levels of both genes were rapidly up-regulated. In both instances, induction by cinnamic acid was greater than that by sorbic acid.

3.2. Effect of substrate concentration on decarboxylation

In theory, *trans* (*E*)-1,3-pentadiene should be produced in equimolar proportion to the sorbic acid applied, provided that sufficient acid had been applied to induce the system and that neither time nor enzymic capacity was limiting. The effect of acid concentration on decarboxylation activity was therefore determined experimentally. Results showed that over 10 h, sorbic acid applied at concentrations up to 1.3 mM was indeed converted into 1,3-pentadiene in equimolar proportion by *A. niger* conidia (Fig. 3). However, increased concentrations of sorbic acid resulted in progressively less 1,3-pentadiene production, and at high concentrations of sorbic acid (approaching the growth inhibitory concentration), the production of 1,3-pentadiene was almost entirely suppressed (Fig. 3). This indicates that the toxic attributes of sorbic acid prevented its own decarboxylation, and that suppression of the decarboxylation resistance mechanism was the determining factor in establishing the overall resistance of *A. niger* conidia to preservatives. In view of this, the toxicity of all substrates tested was pre-determined in YEPD media at pH 4.0 and 10^7 conidia/ml. A substrate concentration of 1 mM was chosen as a non-inhibitory concentration that would permit easy detection of decarboxylation.

3.3. Role of 2,3,4,5,6-pentafluorocinnamic acid in decarboxylation

Any single compound successfully decarboxylated by the Pad-decarboxylation system in *A. niger* whole conidia must of necessity and must be both an inducer of the system and an enzymic substrate. We have previously shown that *padA1* induction occurs transcriptionally (Plumridge et al., 2010) but there is no knowledge of the induction mechanism. Any compound not decarboxylated by conidia could fail as an inducer, or as a substrate, or both. It was therefore necessary to devise a strategy to separate the inducer and substrate functions of individual compounds. 2,3,4,5,6-Pentafluorocinnamic acid is an analogue of cinnamic acid with greater antifungal activity than cinnamic acid itself. Unlike cinnamic acid, pentafluorocinnamic acid was not decarboxylated by *A. niger* conidia. No trace of a 2,3,4,5,6-pentafluorostyrene peak was found in the GCMS data, or indeed of any other fluorinated breakdown product. However, in 6 h cell-free extracts of *A. niger* conidia induced by 1 mM sorbic acid, 2,3,4,5,6-pentafluorocinnamic acid was strongly decarboxylated to 2,3,4,5,6-pentafluorostyrene (Supplementary data Table 1, entry 121). No such activity was found in induced cell-free extracts of the *A. niger* strain AXP2.21a (Δ*padA1*) thus demonstrating that 2,3,4,5,6-pentafluorocinnamic acid was a substrate for the decarboxylase

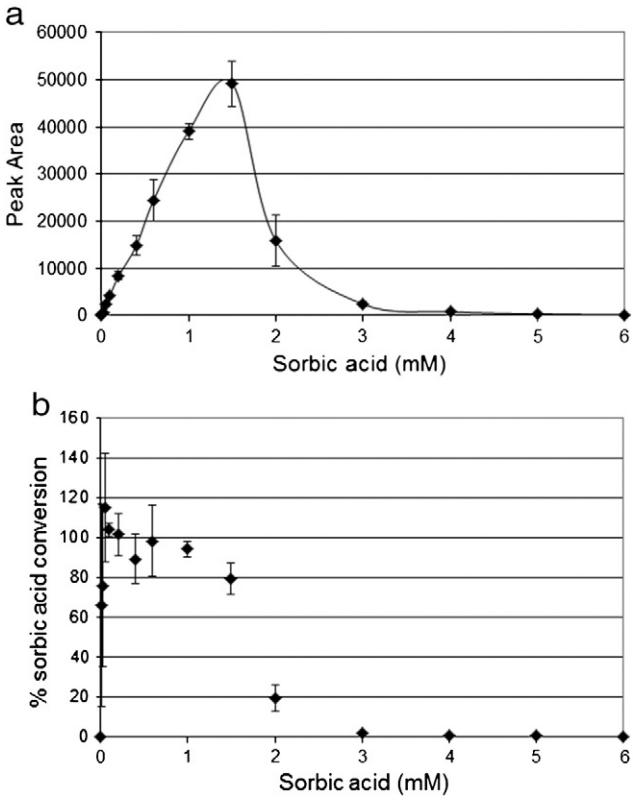


Fig. 3. Conversion of increasing concentrations of sorbic acid into 1,3-pentadiene by germinating conidia of *Aspergillus niger* measured after 10 h, by peak area (Fig. 3a) or % sorbic acid conversion by decarboxylation (Fig. 3b). Volatiles were detected by GCMS of headspace samples. Quantitative conversion of 1 mM sorbic acid to 1,3-pentadiene corresponds to the equilibrated mean control 1,3-pentadiene 1 mM peak area of 41,422.

system. Absence of activity on 2,3,4,5,6-pentafluorocinnamic acid by whole conidia suggests either a lack of acid permeation into the cell (hydrophobic acid permeation is normally by simple diffusion) or a lack of activity as a decarboxylase inducer.

Tests carried out using combinations of cinnamic acid and 2,3,4,5,6-pentafluorocinnamic acid on intact *A. niger* conidia showed that both of these acids had the ability to permeate the cell. It was found that in the presence of 0.01–1 mM cinnamic acid, 2,3,4,5,6-pentafluorocinnamic acid was strongly decarboxylated by whole conidia. In this instance, it may be inferred that cinnamic acid induced the Pad-decarboxylase, which then acted upon both cinnamic and 2,3,4,5,6-pentafluorocinnamic acids yielding a mixture of styrene and 2,3,4,5,6-pentafluorostyrene. Putative competition by the two acids for the active site is revealed in the detail of Fig. 4. In this series of experiments, 2,3,4,5,6-pentafluorocinnamic acid was used throughout at 0.5 mM together with cinnamic acid at a range of concentrations, and decarboxylation was determined at 6 h. Low concentrations of cinnamic acid (0.01 mM) were sufficient to induce the decarboxylase, which then acted on both of the acids but predominantly against the more numerous 2,3,4,5,6-pentafluorocinnamic acid molecules, forming a mixture of pentafluorostyrene and styrene (Fig. 4). Increased concentrations of cinnamic acid progressively increased decarboxylase induction. At equimolar (0.5 mM) acid concentrations, more styrene was formed than 2,3,4,5,6-pentafluorostyrene, indicating acid competition for the active site and greater affinity of the enzyme for cinnamic acid than 2,3,4,5,6-pentafluorocinnamic acid. Higher concentrations of cinnamic acid progressively reduced decarboxylation but affected the decarboxylation of 2,3,4,5,6-pentafluorocinnamic acid to a greater extent (Fig. 4). From this experiment, it was confirmed that the concentration of cinnamic acid required to induce decarboxylation was low (<0.01 mM) but that induction progressively increased up to 1.5 mM. 2,3,4,5,6-Pentafluorocinnamic acid was therefore a substrate

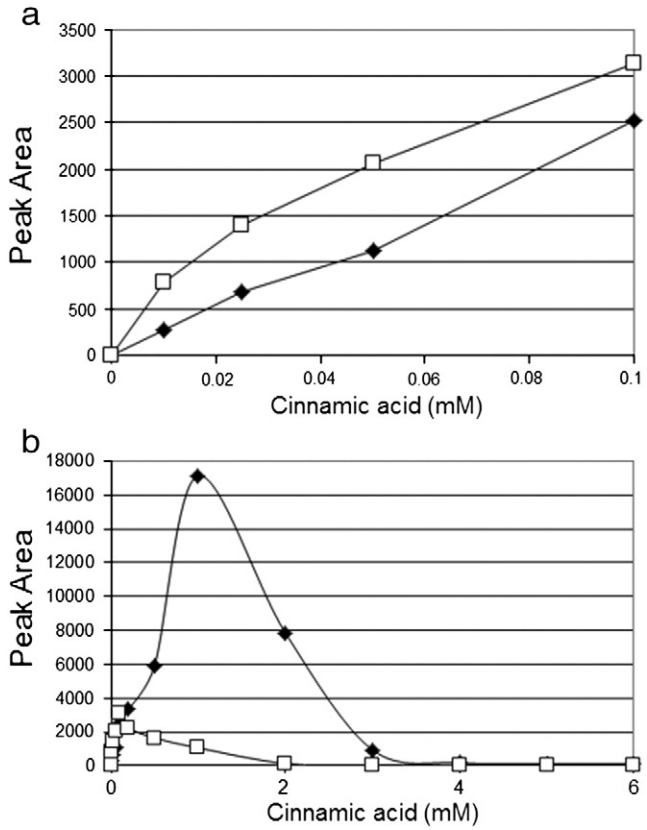


Fig. 4. Decarboxylation of mixtures of cinnamic acid and 2,3,4,5,6-pentafluorcinnamic acid to styrene (closed symbols) and 2,3,4,5,6-pentafluorostyrene (open symbols) by germinating conidia of *Aspergillus niger* measured after 6 h. Experiments contained constant 0.5 mM 2,3,4,5,6-pentafluorocinnamic acid and variable cinnamic acid (Fig. 4a 0–0.1 mM, Fig. 4b 0–6 mM) concentrations. Volatiles were detected by GCMS of headspace samples. Styrene controls, prepared at 1 mM and equilibrated for 10 h, gave a GCMS peak area of 48,046.

for decarboxylation only, not an inducer, a fact confirmed by the lack of transcription of either *padA1* or *ohbA1* (Fig. 2). Thus, 2,3,4,5,6-pentafluorocinnamic acid could be used as a reporter to detect activity of Pad-decarboxylation and *padA1* induction by other compounds, which in themselves may not be substrates for decarboxylation.

Detailed probing of the decarboxylase system and the structural requirements for transcriptional induction of *padA1* were then carried out using 1 mM substrate concentrations against whole conidia, 1 mM substrate concentrations against cell-free extracts after 6 h induction, and 0.5 mM substrate + 0.5 mM 2,3,4,5,6-pentafluorocinnamic acid against whole conidia. Those compounds decarboxylated by whole conidia were both substrates and inducers, whereas those decarboxylated by cell-free extracts were substrates, and those liberating 2,3,4,5,6-pentafluorostyrene were inducers.

3.4. Structural features of substrates for induction and functionality of the Pad-decarboxylation system

A substantial number of potential substrates are listed in Supplementary data Table 1 in order of molecular mass and listed according to the entry number (referred subsequently as SD entry followed by the relevant number, e.g. acrylic acid in SD entry 1 and 2,3,4,5,6-pentafluorocinnamic acid is SD entry 121). These compounds were used to determine the important structural features required of successful substrates for decarboxylation by the Pad system. The carboxylic acid group at C1 in both sorbic acid and cinnamic acid is the hydrophilic head-group of these amphipathic compounds, whereas the remainder of their structures are substantially hydrophobic. As anticipated, any changes made in the level of oxidation at C1 completely removed all decarboxylase activities in *A. niger* conidia. Thus no activity was detected using sorbic alcohol, sorbic aldehyde, cinnamyl alcohol or cinnamaldehyde or cinnamic-acid methyl- or ethyl-esters (SD entries 8;11,36,37,60,77). Ester, aldehyde and alcohol analogues were all shown to be incapable of inducing the decarboxylase system. However, a low level of apparent decarboxylation of these compounds was detected using pre-induced enzyme suggesting that they may be poor, indirect substrates, probably following a low level of oxidation to the corresponding carboxylic acid.

Sorbic acid and cinnamic acid both contain an alkenyl bond between carbons 2 and 3, which has the *trans* (*E*)-configuration (Table 1). This bond is essential. Removal of this alkenyl bond in either of the carboxylic acids abolished all activities as decarboxylase inducer or substrate. The structures of these acids are shown in Table 2. The effect of the C2–C3 alkenyl bond may simply be due to its unsaturation or its geometry, or a combination of both. Analogues of the alkenyl fragment between C2 and C3, such as a triple bond or a cyclopropane ring (e.g. phenylpropiolic acid and 2-phenylcyclopropanecarboxylic acid, SD entries 44,62), did not substitute for the alkene bond. The importance of the *trans* (E)-configuration of the C2–C3 alkene bond was examined by comparing *cis* (*Z*)-2,4-methoxycinnamic acid with its *trans* (*E*)-isomer. The *trans* (*E*)-isomer was active both as an inducer and as a substrate while the *cis* (*Z*)-isomer was inactive (SD entries 79,82).

Sorbic acid also contains alkene unsaturation between C4 and C5 and cinnamic acid is substituted by a phenyl ring at C3. Removal of the C4–C5 unsaturation in sorbic acid again abolished all activity as inducer or substrate for Pad-decarboxylation (Table 2; 2-hexenoic acid). While the *trans* (*E*)-configuration at C4–C5 in sorbic acid is important for activity, cinnamic acid, together with the furan and thiophene analogues shown in Table 2, contain additional or extended unsaturation at C3. This extended unsaturation, however, allows the molecules to assume a similar shape to that found in sorbic acid, which presumably is one reason why the furan and thiophene

Table 1
The relationship between an alkenyl double bond at C2 and activity as a Pad-decarboxylation inducer or substrate in conidia of *Aspergillus niger*. Carbons are numbered from the carboxylic acid as indicated on the structure of sorbic acid.

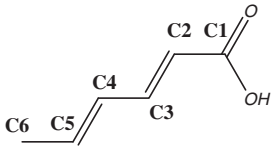
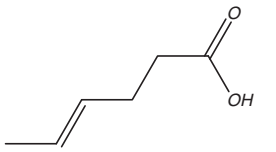
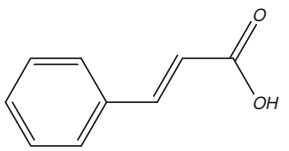
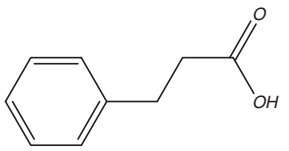
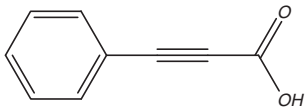
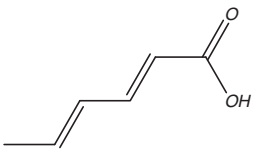
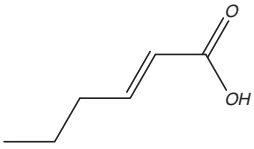
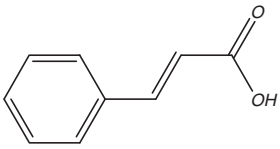
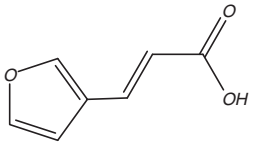
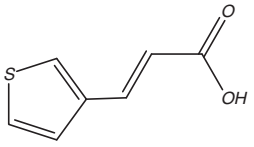
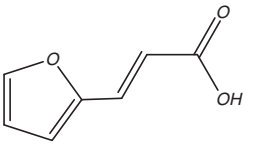
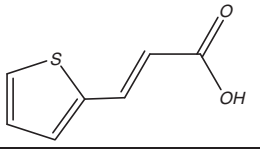
Chemical	Structure	Inducer	Substrate
Sorbic acid		Yes	Yes
Trans (*E*)-4-Hexenoic acid		No	No
Cinnamic acid		Yes	Yes
Hydrocinnamic acid		No	No
Phenylpropiolic acid		No	No

Table 2
The relationship between *trans* (*E*)-unsaturation at C4 and activity as a Pad-decarboxylation inducer or substrate in conidia of *Aspergillus niger*.

Chemical	Structure	Inducer	Substrate
Sorbic acid		Yes	Yes
Trans (*E*)-2-hexenoic acid		No	No
Cinnamic acid		Yes	Yes
Trans (*E*)-3-(3-Furanyl) propenoic acid		Yes	Yes
Trans (*E*)-3-(3-Thienyl) propenoic acid		Yes	Yes
Trans (*E*)-3-(2-Furanyl) propenoic acid		Yes	Yes
Trans (*E*)-3-(2-Thienyl) propenoic acid		Yes	Yes

analogues are successfully decarboxylated as Pad substrates and inducers in whole conidia.

All of the compounds that were found to decarboxylate with high activity carried substituents beyond C5 in their structures. In cinnamic acid, this extension formed part of the aromatic ring and sorbic acid accommodated a methyl group at C5. Removal of this additional substitution at C5 resulted in substantial loss of decarboxylation activity, as demonstrated by the low level of activity against *trans* (*E*)-2,4-pentadienoic acid (SD entry 10). This carboxylic acid contains all of the significant features mentioned previously, and would seem to fit into a site that would accommodate sorbic acid, yet it was decarboxylated poorly, and is particularly poor as an inducer. This feature strongly indicates that a carbon substituent at C5 in sorbic acid is a pre-requisite for induction.

3.5. Size of substrates for the Pad-decarboxylase system

The size requirements for compounds to serve either as inducers or substrates for the Pad-decarboxylase were assessed using a number of sorbic acid analogues containing between 5 and 10 carbon atoms. All of these analogues contained the essential trans (E)-2, trans (E)-4-alkene bonds. All of the analogues examined were found to decarboxylate to their corresponding diene hydrocarbons, but the level of decarboxylation by whole conidia from 2,4-pentadienoic acid, 2,4-nonadienoic acid and 2,4-decadienoic acid was only slight (Fig. 5). High activity comparable to sorbic acid was found with 2,4-heptadienoic acid and 2,4-octadienoic acid indicating that the length of substrates should be between ~6 and 9 Å. Induction by 2,4-pentadienoic acid, 2,4-nonadienoic acid and 2,4-decadienoic acid, as detected using 2,3,4,5,6-pentafluorocinnamic acid, was also considerably lower than with sorbic acid. Decarboxylation in cell-free extracts was less affected, indicating that the structural requirements for induction were more discriminatory than the enzyme active site.

Additional data concerning the overall length of substrate molecules were obtained using a range of 4-substituted cinnamic acid analogues. Thus, 4-methyl-, 4-methoxy- and 4-ethoxy-cinnamic acids were decarboxylated to 4-methylstyrene, 4-methoxystyrene and 4-ethoxystyrene respectively indicating that substrate molecules could be ~9.1 Å in length (SD entries 57, 81, 99). Again, the structural requirements for induction were more discriminatory than the enzyme active site.

The "width" of substrates was also assessed using a variety of substituted cinnamic acids. Single methyl-substitutions at positions 2, 3, 5 and 6 in the aromatic rings of cinnamic acids (SD entries 55, 56, 57), resulted in high levels of activity indicating that the width of substrates at the phenyl ring level could be up to 5.2 Å. Methoxy-substituted cinnamic acids were also decarboxylated (SD entries 79,80,81). Although α-fluorocinnamic acid was efficiently decarboxylated, α-methylcinnamic acid showed lower activity and α-phenylcinnamic acid was not recognised as either substrate or inducer. This observation indicated a substrate width limitation of ca. 3.6 Å at C2. This suggested that width limitation was supported by the observed lowering of decarboxylation in 2′-substituted cinnamic acids, compared with the 3′- and 4′-substituted acids. Reduced decarboxylation and induction were observed using 2′-trifluoromethyl-cinnamic acid and 2′-ethoxy-cinnamic acid (SD entries 97,111).

3.6. Polar and hydrophobic substituted cinnamic acids

Several other substituted cinnamic acids were examined as substrates and inducers of decarboxylation. In general, hydrophobic substitutions in the phenyl ring were decarboxylated successfully. These included fluoro-, chloro-, and bromo-substitutions in any position, and trifluoromethyl substitutions (SD entries 67–69, 85–87, 111–113, 118–120). Difluoro and trifluoro-substituted cinnamic acids were also accepted (SD entries 88–92, 105). All of these substrates were decarboxylated with high efficiency and they served as powerful inducers.

Cinnamic acids substituted by polar/hydrophilic groups, e.g. hydroxyl-, nitro-, or amino-substituents were not accepted (SD entries 63–66, 100–102). The lack of recognition of hydroxycinnamic acids is particularly significant as they include those acids derived from plant cell walls, i.e. coumaric acid, vanillic acid and ferulic acid. These acids have previously been reported as substrates of Pad enzymes of bacterial (van Beek and Priest, 2000) and yeast (Mukai et al., 2010) origin, and so additional tests were carried out. 4-Hydroxycinnamic acid (coumaric acid) at a range of concentrations, 0.1 mM–32 mM, showed no detectable decarboxylation by whole conidia of *A. niger*, or in induced cell-free extracts, or in *padA1*/*ohbA1* transcription (Fig. 1). Furthermore, 4-hydroxycinnamic acid in combination with 2,3,4,5,6-pentafluorocinnamic acid showed a complete lack of induction activity at any concentration. The lack of decarboxylation of 4-hydroxycinnamic acid is unlikely to be caused by size constraints since the larger 4-methoxycinnamic acid was successfully decarboxylated (SD entry 81). Rejection of 4-hydroxycinnamic acid is probably a result of the acidity of the phenolic-hydroxyl moiety. This feature indicates that 4-hydroxycinnamic acid cannot be the natural decarboxylation target of the Pad system in *A. niger*, since it is neither able to activate the system nor to be recognised as a substrate for decarboxylation.

Given the wide variety of carboxylic acids that have been tested in this study, we questioned whether all of the acids are decarboxylated

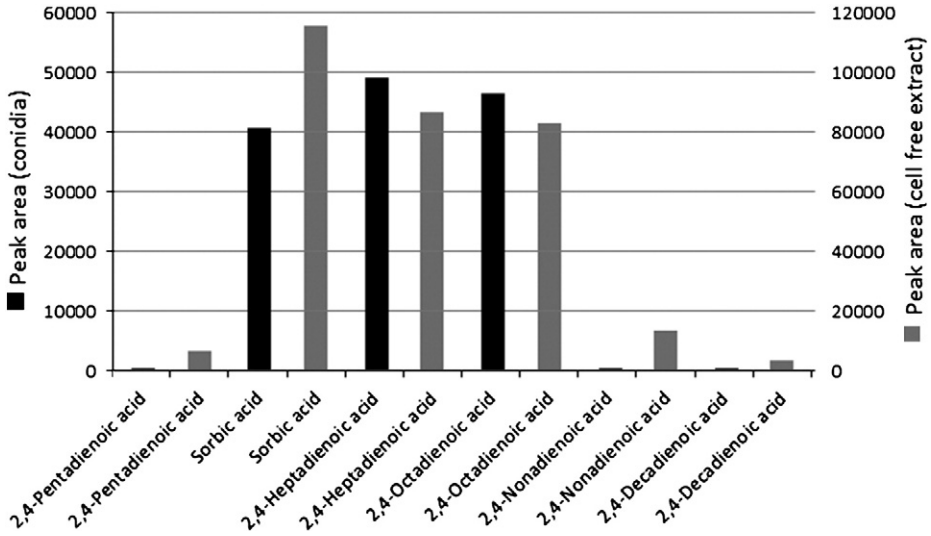


Fig. 5. Decarboxylation of sorbic acid length homologues by germinating conidia (dark histograms) and cell free extracts (light histograms) of *Aspergillus niger*. Volatiles were detected by GCMS of headspace samples, adjusted in volume to maximise sensitivity (0.2 ml conidia; 10 ml cell-free) without overloading GCMS peaks, preventing quantitative comparison of conidia/cell-free extracts. 2,4-pentadienoic acid gave 1,3-butadiene, sorbic acid gave 1,3-pentadiene. 1,3-Pentadiene controls, prepared at 1 mM and equilibrated gave GCMS peak areas of 41,099 (conidia) and 111,780 (cell-free).

by the same enzyme or enzyme system. Therefore, all of the acids found to be successfully decarboxylated by *A. niger* spores in the above study were also tested using *A. niger* AXP6-2.21a (Δ*padA1*) (Plumridge et al., 2008). Interestingly, no decarboxylation of any substrate occurred in the Δ*padA1* strain, thereby demonstrating that only a single decarboxylation system was likely to be involved.

4. Discussion

Saprobic or pathogenic fungi interact with a variety of toxic or inhibitory compounds in their natural environments and therefore require efficient resistance mechanisms to survive. It is a notable feature of resistance mechanisms, that they are often pleiotropic, having sufficient flexibility to accommodate a variety of minor changes in chemical structure, for example, drug pumps conferring antibiotic resistance (Goffeau et al., 1997; Kowlaczkowski and Goffeau, 1997). The Pad-decarboxylation system of *A. niger* investigated here is similarly pleiotropic which is, in itself, an indication that the Pad-decarboxylation system in germinating fungal spores is primarily a resistance mechanism to environmental toxins. We have shown that there are essential features of a high-activity substrate for Pad-decarboxylation that comprise a carboxylic acid, *trans* (*E*)-alkene bonds at the C2 and C4 positions, and a carbon substituent at C5. The minimal structure of a high-activity substrate is therefore *trans* (*E*), *trans* (*E*)-2,4-hexadienoic acid, i.e. sorbic acid. By contrast, the largest structure of a high-activity substrate is represented by a substituted cinnamic acid. The largest scope for variation in these structures is in the hydrophobic portions of the compounds furthest from their carboxyl groups. The flexibility in this region allows alternative heterocyclic ring structures to be used in place of the phenyl ring of cinnamic acid.

A further pointer to the role of the Pad-decarboxylation system comes from comparing its activity in yeasts and moulds. Pad-decarboxylation has previously been shown to occur at high activity in germinating conidia of a variety of *Aspergillus* spp. (Plumridge et al., 2010). It is also widespread in germinating spores of *Penicillium* and *Trichoderma* spp. (Marth et al., 1966; Pinches and Apps, 2007). High activity Pad-decarboxylation can therefore be regarded as common in germinating mould conidia. In contrast, Pad-decarboxylation in yeasts occurs more rarely. Pad1p homologues were found in only 8 out of 23 reported yeast genome sequences, and decarboxylation was observed in only Pad1p-containing species (Stratford et al., 2007; Mukai et al., 2010). Furthermore, when Pad-decarboxylation did occur in yeasts, the activity was low and was insufficient to enhance the resistance to weak acids. It appears most probably that high-activity Pad-decarboxylation is primarily a mould phenomenon, and since the native environment for most yeast species is sugar-rich (typically fruit, flowers and insects), this indicates that the substrates for Pad-decarboxylation are not found in those environments.

The Pad-decarboxylation system was found to occur at high level in germinating conidia, falling to a lower level as hyphae developed (Plumridge et al., 2004). That feature could indicate that Pad-decarboxylation is related to removal of a self-inhibitor of spore germination. Decarboxylation of any self-inhibitor substrate by Pad-decarboxylation would result in the formation of volatile hydrocarbons having unsaturation at positions C1 and C3. However, our examination of volatile compound formation by germinating wild-type and Δ*padA1* strains of *A. niger* gave no evidence for such volatiles (unpublished data). Furthermore, evidence of gene induction shows that the *padA1* and *ohbA1* genes were poorly transcribed in germinating spores unless exogenous acids were added (Plumridge et al., 2010). We therefore conclude that the Pad-decarboxylation system is unlikely to function in the removal of self-inhibition in germinating spores.

After analysis of the range of Pad-decarboxylation substrates, the most probable naturally occurring substrates appear to be sorbic acid and cinnamic acid. Sorbic acid can be obtained from the whitebeam tree, *Sorbus aria*, and from berries of the mountain ash, *Sorbus aucuparia*. Cinnamic acid is more common, and has been reported in balsam, storax and cocoa leaves, in addition to oils of basil and cinnamon (Burdock, 2002). In oil of cinnamon, cinnamic acid forms a large proportion, together with its corresponding aldehyde, cinnamaldehyde. The essential oil of cinnamon is a long-recognised anti-fungal agent (Myers, 1927) and it is probable that the essential oil is produced to prevent plant infection by fungi. It can be speculated that cinnamic acid was at one time more-commonly produced in plants, or that moulds such as *Penicillium*, *Aspergillus* and *Trichoderma* spp. have evolved from moulds infecting or recycling cinnamic-acid-producing plants.

In a previous publication (Plumridge et al., 2010) we reported that Pad-decarboxylation in *A. niger* required activity by two genes, *padA1* and *ohbA1* and this was confirmed in *S. cerevisiae* (Mukai et al., 2010). How these gene products interact is not yet known in fungi, but homologues are known to interact in prokaryotes. The *E. coli*

homologues of *padA1* and *ohbA1* are *UBIX* and *UBID* respectively, and both are required in the synthesis of coenzyme Q. Yeast *PAD1* can functionally replace *ubiX* in *E. coli* (Gulmezian et al., 2007); yeast deleted for *pad1* has normal levels of coenzyme Q but lacks Pad-decarboxylation. The synthesis of coenzyme Q in many bacteria has been shown to require the presence of both Pad1p/UbiXp and Ohb1p/UbiDp (Rangarajan et al., 2004; Lupa et al., 2005), clearly showing a level of divergence in function. Whilst it has been reported that some prokaryotes can decarboxylate hydroxybenzoic and hydroxycinnamic acids (Cavin et al., 1998), these compounds are not substrates for Pad-decarboxylation in the fungi studied here. Instead, they are rejected as enzyme substrates and fail to induce transcription of the relevant genes. It would appear that the two putative decarboxylases, Pad1p/UbiXp and Ohb1p/UbiDp are both required for coenzyme Q synthesis in bacteria (and are able to act on 2-hydroxybenzoic acid) and that both are required for Pad-decarboxylation in fungi (but not acting on 2-hydroxybenzoic acid and not affecting coenzyme Q).

X-ray crystallography studies on the *E. coli* Pad1p/UbiXp (Rangarajan et al., 2004) have shown that Pad-type proteins associate into oligomers with a trimer forming the common structural unit although 12-mer assemblies have been predicted for Pad from both *E. coli* (Rangarajan et al., 2004; PDB entry 1sbz) and *Aquifex aeolicus* (PDB entry 2ejb). The active site of the *E. coli* enzyme was identified at the interface of the three Pad monomers, although the interaction with Ohb/UbiD was not examined. The role of OhbA1 in fungi is clearly essential in Pad-decarboxylation but does not appear to involve 2-hydroxybenzoic acid decarboxylation. A structural role for the OhbA1 in a hetero-oligomeric OhbA1/PadA1 protein complex may be possible, such as maintaining PadA1 oligomers in the correct conformation, but a physical or mechanistic interaction between PadA1 and OhbA1 remains speculative. In view of the lack of firm data, it would seem inappropriate at this time to speculate upon the chemical mechanism of decarboxylation by the Pad-decarboxylation system.

From our knowledge of what structural features are required to be a substrate for Pad-decarboxylation, a number of non-competitive enzyme inhibitors have been found which prevent enzyme activity and decrease mould resistance to sorbic acid. These are typically 2,4-unsaturated aldehydes that conform to the dimensions defined for substrates (Archer et al., 2008). We speculate that the chemically reactive aldehyde moiety causes enzyme damage by covalently bonding, preventing further activity. It is interesting to note that oil of cinnamon contains not only cinnamic acid but also cinnamaldehyde (Burdock, 2002), one of the key inhibitors of Pad-decarboxylation. It appears to indicate not only synthesis of cinnamic acid by plants as an inhibitor of mould infection but also possibly the synthesis of the aldehyde to combat the mould's mechanism of resistance to cinnamic acid.

Supplementary materials related to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2012.06.007.

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References

Anon, 1995. EC directive 95/2/EC; food additives other than colours and sweeteners. EC Official Journal (L61), 1–40 (March 18th).
Archer, D.B., Stratford, M., Plumridge, A., 2008. Preservative. Patent number WO2008/149102 A2. PCT/GB2008/001935. Published 11/12/08.
Belin, P., Le Du, M.H., Fielding, A., Lequin, O., Jacquet, M., Charbonnier, J.-B., Lecoq, A., Thai, R., Courcon, M., Masson, C., Dugave, C., Genet, R., Pernodet, J.-L., Gondry, M., 2009. Identification and structural basis of the reaction catalysed by CYP121, an essential cytochrome P450 in *Mycobacterium tuberculosis*. Proceedings of the National Academy of Sciences, USA 106, 7426–7431.
Bos, C.J., Debets, A.J.M., Swart, K., Huybers, A., Kobus, G., Slakhorst, S.M., 1988. Genetic analysis and construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Current Genetics 14, 437–443.
Breeuwer, P., de Reu, J.-C., Drocourt, J.-L., Rombouts, F.M., Abee, T., 1997. Nonanoic acid, a fungal self-inhibitor, prevents germination of *Rhizopus oligosporus* sporangiospores by dissipation of the pH gradient. Applied and Environmental Microbiology 63, 178–185.
Burdock, G.A., 2002. Fenaroli's Handbook of Flavor Ingredients, 4th Edn. CRC Press, Boca Raton, London, New York, Washington.
Casas, E., Valderrama, M.J., Peinado, J.M., 1999. Sorbate detoxification by spoilage yeasts isolated from marzipan products. Food Technology and Biotechnology 37, 87–91.
Casas, E., de Ancos, B., Valderrama, M.J., Cano, P., Peinado, J.M., 2004. Pentadiene production from potassium sorbate by osmotolerant yeasts. International Journal of Food Microbiology 94, 93–96.
Cavin, J.-F., Dartois, V., Divies, C., 1998. Gene cloning, transcriptional analysis, purification and characterization of phenolic acid decarboxylase from *Bacillus subtilis*. Applied and Environmental Microbiology 64, 1466–1471.
Chitarra, G.S., Abee, T., Rombouts, F.M., Posthumus, M.A., Dijksterhuis, J., 2004. Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. Applied and Environmental Microbiology 70, 2823–2829.
Ciulli, A., Abell, C., 2007. Fragment-based approaches to enzyme inhibition. Current Opinions in Biotechnology 18, 489–496.
Ciulli, A., Scott, D.E., Ando, M., Reyes, F., Saldanha, S.A., Tuck, K.L., Chirgadze, D.Y., Blundell, T.L., Abell, C., 2008. Inhibition of *Mycobacterium tuberculosis* pantothenate synthetase by analogues of the reaction intermediate. ChemBioChem 9, 2606–2611.
Clausen, M., Lamb, C.J., Megnet, R., Doerner, P.W., 1994. *PAD1* encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. Gene 142, 107–112.
Garrett, M.K., Robinson, P.M., 1969. A stable inhibitor of spore germination produced by fungi. Archives fur Mikrobiology 67, 370–377.
Goffeau, A., Park, J., Paulsen, I.T., Jonniaux, J.-L., Dinh, T., Mordant, P., Saier, M.H., 1997. Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of yeast open reading frames within the major facilitator superfamily. Yeast 13, 43–54.
Gulmezian, M., Hyman, K.R., Marbois, B.N., Clarke, C.F., Javor, G.T., 2007. The role of UbiX in *Escherichia coli* coenzyme Q biosynthesis. Archives of Biochemistry and Biophysics 467, 144–153.
Hann, M.M., Leach, A.R., Harper, G., 2001. Molecular complexity and its impact on the probability of finding leads for drug discovery. Journal of Chemical Information and Computer Sciences 41, 856–864.
Hung, A.W., Silvestre, H.L., Wen, S., Ciulli, A., Blundell, T.L., Abell, C., 2009. Application of fragment growing and fragment linking to the discovery of inhibitors of *Mycobacterium tuberculosis* pantothenate synthetase. Angewandte Chemie International Edition 48, 8452–8456.
Kinderlerler, J.L., Hutton, P.V., 1990. Fungal metabolites of sorbic acid. Food Additives and Contaminants 7, 657–669.
Kobayashi, Y., Nakano, M., Kumar, G.B., Kishihara, K., 1998. Efficient conditions for conversion of 2-substituted furans into 4-oxygenated 2-enoic acids and its application to synthesis of (+)-aspicilin. Journal of Organic Chemistry 63, 7505–7515.
Kowlaczkowski, M., Goffeau, A., 1997. Active efflux by multidrug transporters as one of the strategies to evade chemotherapy and novel practical implications of yeast pleiotropic drug resistance. Pharmacology and Therapeutics 76, 219–242.
Kurogochi, S., Tahara, S., Mizutani, L., 1975. Fungal reduction of C6 α, β unsaturated carboxylic acids. Agricultural and Biological Chemistry 39, 825–831.
Lachance, M.-A., 1989. Yeast selection in nature. In: Panchal, C.J. (Ed.), Yeast Strain Selection. Marcel Dekker Inc., New York, pp. 21–41.
Larkin, A., Olivier, N.B., Imperiali, B., 2010. Structural analysis of WbpE from *Pseudomonas aeruginosa* PAO1: a nucleotide sugar aminotransferase involved in o-antigen assembly. Biochemistry 49, 7227–7237.
Liu, Z.L., Palmquist, D.E., Ma, M., Liu, J., Alexander, N.J., 2009. Application of a master equation to quantitative mRNA analysis using qRT-PCR. Journal of Biotechnology 143, 10–16.
Lupa, B., Lyon, D., Gibbs, M.D., Reeves, R.A., Wiegel, J., 2005. Distribution of genes encoding the microbial non-oxidative reversible hydroxyacrylic acid decarboxylases/phenol carboxylases. Genomics 86, 342–351.
Marth, E.H., Capp, C.M., Hasenzah, L., Jackson, H.W., Hussong, R.V., 1966. Degradation of potassium sorbate by *Penicillium* species. Journal of Dairy Science 49, 1197–1205.
Mukai, N., Masaki, K., Fujii, T., Kawamukai, M., Iefuji, H., 2010. *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering 109, 564–569.
Murray, C.W., Verdonk, M.L., 2002. The consequences of translational and rotational entropy lost by small molecules on binding to proteins. Journal of Computer-Aided Molecular Design 16, 741–753.
Myers, H.B., 1927. An unappreciated fungicidal action of certain volatile oils. Journal of the American Medical Association 89, 1834–1836.
Olivier, N.B., Imperiali, B., 2008. Crystal structure and catalytic mechanism of PglD from *Campylobacter jejuni*. Journal of Biological Chemistry 283, 27937–27946.
Pinches, S.E., Apps, P., 2007. Production in food of 1,3-pentadiene and styrene by *Trichoderma* species. International Journal of Food Microbiology 116, 182–185.
Plumridge, A., Hesse, S.J.A., Watson, A.J., Lowe, K.C., Stratford, M., Archer, D.B., 2004. The weak-acid preservative, sorbic acid, inhibits conidial germination and mycelial growth of *Aspergillus niger* through intracellular acidification. Applied and Environmental Microbiology 74, 550–552.
Plumridge, A., Stratford, M., Lowe, K.C., Archer, D.B., 2008. The weak-acid preservative, sorbic acid, is decarboxylated and detoxified by a phenylacrylic acid decarboxylase,

PadA1, in the spoilage mould *Aspergillus niger*. Applied and Environmental Microbiology 74, 550–552.

Plumridge, A., Melin, P., Stratford, M., Novodvorska, M., Shunburne, L., Dyer, P.S., Roubos, J.A., Menke, H., Stark, J., Stam, H., Archer, D.B., 2010. The decarboxylation of the weak-acid preservative, sorbic acid, is encoded by linked genes in *Aspergillus* spp. Fungal Genetics and Biology 47, 683–692.

Rangarajan, E.S., Li, Y., Iannuzzi, P., Tocilj, A., Hung, L.-W., Matte, A., Cygler, M., 2004. Crystal structure of a dodecameric FMN-dependent, UbiX-like decarboxylase (Pad1) from *Escherichia coli* 0157:H7. Protein Science 13, 3006–3016.

Scott, D.E., Dawes, G.J., Ando, M., Abell, C., Ciulli, A., 2009. A fragment-based approach to probing adenosine recognition sites by using dynamic combinatorial chemistry. ChemBioChem 10, 2772–2779.

Stratford, M., Anslow, P., 1997. A preservative and flavouring system for beverages based on cinnamic acid and its derivatives. Patent application filed 28th October 1997, WO 99/21433.

Stratford, M., Plumridge, A., Archer, D.B., 2007. Decarboxylation of sorbic acid by spoilage yeasts is associated with the *PAD1* gene. Applied and Environmental Microbiology 73, 6534–6542.

van Beek, S., Priest, F.G., 2000. Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. Applied and Environmental Microbiology 66, 5322–5328.