

Extrolites of *Wallemia sebi*, a very common fungus in the built environment

Abstract *Wallemia sebi* has been primarily known as a spoilage fungus of dried, salted fish and other foods that are salty or sweet. However, this fungus is also very common in house dust. The health effects of chronic exposure to mold and dampness are known to be associated with both allergens and various inflammatory compounds, including the secondary metabolites of building associated fungi and their allergens. IgE sensitization to *W. sebi* has been long reported from housing and occupational exposures. However, its allergens have not been described previously. Strains from food have been reported to produce a number of compounds with modest toxicity. Strains from the built environment in Canada produced a number of metabolites including the known compound wallemine and a new compound 1-benzylhexahydroimidazo [1,5- α] pyridine-3,5-dione which we call wallimidione. Based on an *in silico* analysis, wallimidione is likely the most toxic of the metabolites reported to date from *W. sebi*. We found that the primary human antigen of *W. sebi* is a 47 kDa excreted cellulase present in high concentrations in *W. sebi* arthrospores. This species is a basidiomycete and, unsurprisingly, the antigen was not found in extracts of other fungi common in the built environment, all ascomycetes.

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Practical Implications

Wallemia sebi is a very common fungus in houses worldwide but relatively more so in north temperate climates. It is well known as a contaminant of salty and sweet foods. Exposure is known to result in IgE sensitization from domestic and workplace exposure, and some metabolites have been reported. This work provides the sequence of the primary human antigen and describes the metabolites from damp building strains including a new compound. Of the atopic patient sera we examined, the percentage with antibodies to the antigen was similar to that of the most common species of *Penicillium* in damp buildings. An important implication of the work is that studies damp building fungi need to use methods capable of detecting this organism more reliably than in the past.

Introduction

More than a century ago, *Wallemia sebi* was studied because it grew on and hence spoiled dried salted fish (Frank and Hess, 1941). It also spoils foods that are sweet (cakes, dates, jams, fruits), salty (fish, meat, peanuts), and/or dry (cereal, bread, rice; Samson et al., 2010). Currently, three species of *Wallemia* are recognized (Zalar et al., 2005). The fungus is xerophilic, that is, growth is independent of the solute used to increase the osmotic pressure of the medium (Wheeler et al., 1988). Japanese and some European researchers first demonstrated that *W. sebi* was abundant in settled dust in homes (Lustgraaf, 1978; Sakamoto et al., 1989; Takahashi, 1997). There are now many studies on its occurrence in settled dust in most temperate areas. *W. sebi* is frequently detected in house dust in Canada,

USA, and Western Europe (Amend et al., 2010; Miller and Day, 1997; Nonnenmann et al., 2012). Sensitization to *W. sebi* was first reported in Japan (Sakamoto et al., 1989). This species is known to elicit an IgE response in humans (Simon-Nobbe et al., 2007). From ca. 1800 children aged 3 to 14 in Germany, 360 (20%) had IgE sensitization to *W. sebi* (Kolossa-Gehring et al., 2007). Occupational allergy to *W. sebi* has also been reported to farmers in Europe (Lappalainen et al., 1998; Reboux et al., 2001).

Three recent reviews concluded that interventions that combine elimination of moisture intrusion and leaks and removal of moldy items help to reduce mold exposure and respiratory symptoms and new onset asthma (Krieger et al., 2010; Mendell et al., 2011; Quansah et al., 2012). This is also the position of government agencies in the US and Canada and of the

World Health Organization (AIHA, 2013; Health Canada, 2007; NIOSH, 2012; WHO, 2009). The health effects of mold in buildings are associated with both protein allergens that may not be species specific (Cramer, 2011). Further, at inhalation exposures that can occur in buildings with mold problems, metabolites present in spore and mycelial fragments have been shown to affect lung biology *in vivo* (Miller et al., 2010). Comprehensive information is therefore needed on the biologically active products of common damp building fungi.

Wallemia sebi produces the metabolites walleminol and wallemione (Frank et al., 1999). Walleminol has an LD₅₀ of 40 µg/ml for brine shrimp and a minimum inhibitory dose of 50 µg/ml for rat liver cells (Wood et al., 1990). Other metabolites that have been reported from *W. sebi* include wallemia A and C (Badar et al., 1972; Ito et al., 1981) and the azasteroid UCA1064-B. This compound demonstrated weak activity against *Saccharomyces cerevisiae*, Gram-positive bacteria and was cytotoxic to HeLa cells (IC₅₀, 15 µM; Takahashi et al., 1993). A cyclopentanopyridine alkaloid with modest activity to *Enterobacter aerogenes* and weakly cytotoxic was reported from a marine isolate (Peng et al., 2011). There are no reports on metabolites produced by strains of *W. sebi* from the USA or Canada. Further, despite the ubiquity of *Wallemia* in house dust and long awareness of its sensitizing potential, no allergens have been reported. The purpose of this study is to describe secondary metabolites of *W. sebi* strains isolated from the built environment and the human antigen of this prominent indoor fungus.

Materials and methods

Cultures

Strains of *W. sebi* were isolated from indoor environments in the Netherlands (CBS 463.97 CBS 112378; Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands), Ontario (DAOM 226641, DAOM 226642, DAOM 242570, DAOM 242571; National Mycological Herbarium at the Department of Agriculture, Ottawa, ON, Canada), northern Saskatchewan (UAMH 7897; University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada) and from Paracel Laboratory, Ottawa. Cultures were aseptically transferred to sterile 2% yeast extract (Difco), 20% sucrose agar plates and slants. These were incubated at 25°C until sufficient growth was observed. After this incubation period, plates and slants were sealed with Parafilm and stored at 4°C until required.

Metabolite screening

The *W. sebi* strains described above were screened for metabolite production as follows. A slant of each was

macerated in sterile distilled deionized water using a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON, Canada). The resulting suspension was used to inoculate (5% v/v) Roux bottles containing 200 ml of sterile 2% yeast extract media supplemented with 20% sucrose. The flasks were incubated at 25°C in the dark for 6 weeks. All strains produced similar metabolite patterns, but UAMH 7897 produced the highest yield of metabolites. For further studies, the culture was inoculated in twenty 250-ml Erlenmeyer flasks containing 50 ml of 2% yeast extract and 20% sucrose sterile liquid media. After 1 week, individual starter cultures were macerated and aseptically transferred to Glaxo bottles containing 1 l of the same media. These were left to incubate for 6 weeks at 25°C.

The liquid culture was separated from the cells by vacuum filtration through a Whatman #4 filter paper. The cells were frozen, lyophilized and stored in the freezer at -20°C, whereas the culture filtrate volume and pH were recorded. The culture filtrates were saturated with NaCl, exhaustively extracted with ethyl acetate (EtOAc), passed through a Whatman #1 filter paper, and anhydrous sodium sulfate prior to being dried by rotary evaporation. The dry crude extract was redissolved in a minimal amount of HPLC grade methanol, filtered through a 25 mm PTFE (0.2 µm) syringe filter, and dried under a gentle stream of nitrogen gas.

The crude extract (4.6 g) was initially dissolved in a minimal amount of CHCl₃ and a few drops of HPLC grade MeOH to aid in solubilization. The extract was applied to a silica gel column (40–63 µm), and metabolites were separated using a MeOH-CHCl₃ gradient increasing from 0 to 10% MeOH in 1% increments followed by flushing with 20 and 50% MeOH in CHCl₃. Column fractions were screened by TLC (0.2 mm Silica gel 60 F₂₅₄ pre-coated) using methanol (10% v/v) in chloroform (MeOH-CHCl₃) and visualized under both long-wave (365 nm) and short-wave (254 nm) light. Like fractions were combined and dried by rotary evaporation.

Each dry column fraction was dissolved in 2 ml of HPLC grade methanol and analyzed by liquid chromatography-ultraviolet-mass spectrometry (LC-UV-MS). This was performed using a Waters 2795 separations module, Waters 996 diode array detector, and Micromass Quattro LC mass spectrometer. Compounds were separated by a Phenomenex Gemini C₁₈ (150 × 4.60 mm, 3 µm, 110 Å) column (Torrance, CA, USA) using a mobile phase consisting of acetonitrile-water (ACN-H₂O) with formic acid (FA) [0.1%, (v:v)]. The solvent gradient was linear programmed from 5 to 100% ACN over 13 min with a flow rate of 1.0 ml/min.

Metabolites were purified from silica gel column fractions employing semipreparative HPLC using a Phenomenex Luna C₁₈ (250 × 10.00 mm, 5 µm, 100 Å) column (Torrance, CA, USA) and a mobile

phase consisting ACN-H₂O. The gradient was linear programmed from 10 to 100% ACN over 20 min with a flow rate of 4 ml/min. Additional cleanup of metabolites was achieved using the similar HPLC conditions depending on the metabolite.

Metabolite NMR spectra were obtained on a Bruker Avance 400 Spectrometer (Milton, ON, Canada) at 400.13 (¹H) and 100 MHz (¹³C) using a 5 mm auto-tuning broadband probe with a Z-gradient. Secondary metabolites were dissolved in CD₃CN (δ H 1.94 and δ C 118.7/1.39) or CD₃OD (δ H 3.30 and δ C 49.0) (CDN Isotopes, Point Claire, QC, Canada) and were referenced to the solvent peak. Chemical shifts were assigned based on ¹H, ¹³C, ¹H/¹H correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and distortionless enhancement by polarization transfer (DEPT-135) spectra utilizing standard Bruker pulse sequences. Metabolite high-resolution mass spectrometry data were acquired on a system equipped with an Agilent 1100 binary pump connected to an Agilent G1969A LC/MSD TOF HRMS. Compounds were separated on a Phenomenex Zorbax C₁₈ (50 × 3.0 mm, 1.8 μ m) column using a mobile phase consisting of ACN-H₂O with FA [0.1%, (v:v)]. The gradient was linear programmed from 10 to 95% ACN over 4 min at a flow rate of 1 ml/min. UV spectra of all major metabolites were obtained using a Varian Cary 3 UV-visible spectrophotometer scanning from 190 to 800 nm. Optical rotations were determined using an Autopol IV polarimeter (Rudolph Analytical, Hackettstown, NJ, USA).

Antigen discovery

The same media and fermentation conditions utilized for *W. sebi* metabolite production described above were also used to screen the culture filtrate for antigenic proteins. However, cultures were incubated until sufficient growth was observed in Roux bottles, 7–14 days depending on the strain. Following the incubation period, the culture filtrate was filtered through cheesecloth to separate it from the mycelium. The mycelium was rinsed with ultrapure water, frozen, lyophilized and stored at –20°C.

Within 2 h of harvesting, NaCl, final concentration of 100 mM, and protease inhibitor [0.05% (v/v); P8215, Sigma, Markham, ON, Canada] were added to the crude culture filtrate. The solution was centrifuged at 30 000 × *g* for 30 min at 4°C. The supernatant was subsequently filtered through cheesecloth to ensure all cellular debris was removed. The culture filtrate was concentrated 50 times with a Hydrosart membrane with a 10 000 kDa cut off (Vivaflo 200 concentrator Sartorius AG, Göttingen, Germany) at a flow rate of 10 ml/min for 12 h to obtain extracellular proteins. During the filtrate concentration, a buffer exchange

(50 mM Tris-HCl buffer, pH 9.0) was performed and the final sample was stored at –20°C until required.

Arthrospore extracts were produced by transferring 10 mg of *W. sebi* arthrospores to a plastic vial with a 3/8 methacrylate bead (ATS Scientific Inc., Burlington, ON, Canada) that was milled for 30 min twice using a Spex-Certiprep mixer mill (Model 5100, Metuchen, NJ, USA). Arthrospore fragments were suspended in 1 ml of 0.5 M Tris buffer with 0.05% Tween 20 (TBST, pH 7.4), and the mixture was sonicated for 1 h using a VWR Scientific Products Aquasonic sonicator (Model 50T, Mississauga, ON, Canada). After sonication, the resulting spore suspension was transferred to a 10 kDa molecular weight cut-off Amicon Ultra-15 centrifuge tube (Millipore, Billerica, MA, USA) and 10 ml of TBST (pH 7.4) was added. The arthrospore solution was centrifuged at 4500 *g* for 30 min at 4°C, down to a volume of 1 ml. The supernatant was subsequently transferred to a 1.5 ml plastic microtube and kept on ice until use in a capture ELISA (1–2 h maximum).

Screening for maximum response to the culture filtrate was performed using ELISA and immunoblotting as previously described by Provost et al. (2013). Sera from 84 atopic patients geographically distributed across North America were obtained from a clinical laboratory for routine and non-routine allergy testing (IBT Reference Laboratory, Lexena, KS, USA). The response to various fungi was tested using the Pharmacia ImmuncAP[®] reagents (Halsey et al., 2000). Human sera were used as the primary antibody for both ELISA and immunoblot tests. The IgG reactive protein bands were documented, and a 47 kDa protein was selected for further work.

To confirm that the 47 kDa protein was antigenic, enhanced anti-*W. sebi* rabbit polyclonal antibodies (RpAbs) were produced by Cedarlane Cedarlane Laboratories Limited (Hornby, ON, Canada, meets the requirements of the Canadian Council on Animal Care). Two rabbits were initially injected with 0.5 mg of the purified 47 kDa protein and boosted three times at 21-day intervals with 0.5 mg of the same protein. Antibody purification was carried out using a protein G column (GE Health Care, Life Sciences, Baie d'Urfe, QC, Canada) according to Wilson et al. (2009).

Protein characterization

The isoelectric point (pI) was measured using a Protean IEF system (Bio-Rad, Mississauga, ON, Canada) focusing for 40 000 V h. The protein sample was applied using active rehydration to pH gradient (IPG) strips (Bio-Rad) with a pH range of 5–8 and 3–10. IPG strips were then silver stained to visualize the target protein. The pI value was calculated according to the percentage of migration from the acidic end of the strip.

To determine the glycosylation status of the target 47 kDa protein, it was separated by SDS-PAGE and

stained with the GelCode glycoprotein staining kit (Pierce, Rockford, IL, USA) with both positive and negative glycoprotein controls and immersed in CBB according to Provost et al. (2013). The gel was fixed in 100 ml of 50% methanol for 30 min and then washed with 100 ml of 3% acetic acid for 10 min twice. All incubation periods were carried out at room temperature on an orbital shaker set at 60 RPM. The gel was transferred to 25 ml of oxidizing solution (Thermo Fisher Scientific, Ottawa, ON, Canada). The gel was washed three times for 5 min in 3% acetic acid and subsequently transferred to 25 ml of glycoprotein staining reagent (Thermo Fisher Scientific) for 15 min. After staining, the gel was transferred to 25 ml of reducing solution (Thermo Fisher Scientific), gently agitated for 5 min, rinsed three times with 3% acetic acid and once with distilled deionized water. The protein bands were compared with the control samples to determine the glycosylation status of the protein.

The target protein was deglycosylated using a trifluoromethanesulfonic acid (TFMS) treatment following a procedure similar to Luo et al. (2010). Prior to deglycosylation, the target 47 kDa protein was desalted using a 5 ml G-25 column (GE Healthcare Life Sciences) and freeze-dried. The lyophilized protein (100 μ g) and the lyophilized positive control (100 μ g horseradish peroxidase, Thermo Fisher Scientific) were placed in separate glass vials, and 45 μ l of pre-cooled TFMS was added to each at 4°C. Each solution was mixed until dissolved and kept at -20°C in an ethanol-dry ice bath for 4 h with occasional shaking. One fifty microliter of pre-cooled 60% pyridine-water was added to each of the vials. Desalting was carried out using a Microcon YM-10 filter (Millipore). The resulting deglycosylated protein samples were separated by SDS-PAGE and stained with CBB as described above.

A 47 kDa protein fraction was separated by 2-D gel electrophoresis, digested with trypsin, and identified according to Twine et al. (2005). Briefly, proteins from the culture filtrate were separated using linear pH 4–7, 7 cm IPG strips (Bio-Rad). Sixty microgram of protein extract was diluted with a solubilization buffer consisting of 4% CHAPS, 2% SB3-10, 0.5% [v/v] pH 3–10 Biolytes (Bio-Rad), and 0.003% [v/v] Orange G. Proteins were loaded onto the IPG strips by in-gel rehydration overnight. Isoelectric focusing was achieved using a Protein IEF Cell (Bio-Rad) using the following conditions: 200 V for 30 min, 500 V for 30 min, 2 h ramp to 6 500 V, and 3 h of focusing at 6 500 V for a total of 20 000–25 000 V h. IPG strips were equilibrated for roughly 30 min in equilibration buffer consisting of 2% SDS, 50 mM Tris/HCl pH 8.8, 6 M urea, 30% glycerol, and 1% DTT followed by the same solution replacing DTT with 4% iodoacetamide. Each IPG strip was quickly rinsed with SDS running buffer (Bio-Rad) and placed onto 10% polyacrylamide gel (8 × 10 cm) with 1% agarose and bromophenol blue

overlay. Gels were run in the second dimension using a PowerPac 1000 (Bio-Rad) at 150–200 V for roughly 45 min. Gels were fixed with 50% ethanol and 5% acetic acid and subsequently stained with silver nitrate to visualize proteins. Protein spots of interest were manually excised from the silver stained gel, destained with 15 mM potassium hexacyanoferrate, and 50 mM sodium thiosulfate. Each spot was individually digested with 20 ng/ μ l trypsin (Promega, Madison, WI, USA) in 50 mM ammonium biocarbonate at 37°C overnight.

Peptides were extracted from gel plugs with 5% ACN in 1% acetic acid, separated by nano-liquid chromatography using a 'CapLC' capillary chromatography system (Waters), and analyzed by tandem mass spectrometry using a QTOF Ultima hybrid quadrupole time-of-flight mass spectrometer (Waters). Tryptically digested peptides were injected on a reverse-phase Pep-Map C₁₈ nano-column (75 μ m × 150 mm) (Dionex, Gloucester, ON, Canada) and resolved by a linear gradient programed from 5% to 75% ACN with 0.12% FA over 30 min at a flow rate of ~350 nl/min. Peptide tandem MS spectra were obtained on the most abundant doubly, triply, and quadruply charged ions. Mascot Daemon (Matrix Science) was utilized to search the genome strain *Wallemia sebi* CBS 633.66 (Padamsee et al., 2012). The antigenic protein was identified based on matching peptide sequences derived from the MS/MS spectra. MS/MS spectra of peptides were accessed based on a Mascot score >30 and by manual *de novo* sequencing.

Results

Metabolite characterization

All the strains tested produced the metabolites discussed in similar amounts. Isolations and characterizations were carried out with cultures of *W. sebi* clade 3 UAMH 7897, using the methods described above. Wallemineone (Figure 1; **1**), tryptophol (**2**), tryptophol acetate (**3**), phenylacetic acid (**4**), and *p*-hydroxybenzoic acid (**5**) were isolated and characterized by NMR, mass spectroscopy, UV, OR, and comparisons to the literature. The proton and carbon spectra for wallemineone isolated here matched the data of Frank et al. (1999). The presence of tryptophol and phenylacetic acid was confirmed by authentic standards (Sigma).

1-benzylhexahydroimidazo [1,5- α] pyridine-3,5-dione, hereafter wallimidione, (**6**) was isolated as a light yellow oil with the molecular formula C₁₄H₁₆N₂O₂ determined by HRESIMS with *m/z* 245.1293 [M+H]⁺ (calculated for 245.1290). The UV spectrum displayed absorption maxima at 275, 248, and 220 nm, indicating the presence of a conjugated system within the molecule. Examination of the ¹H and ¹³C NMR data (Table 1) displayed three sp² quaternary carbons

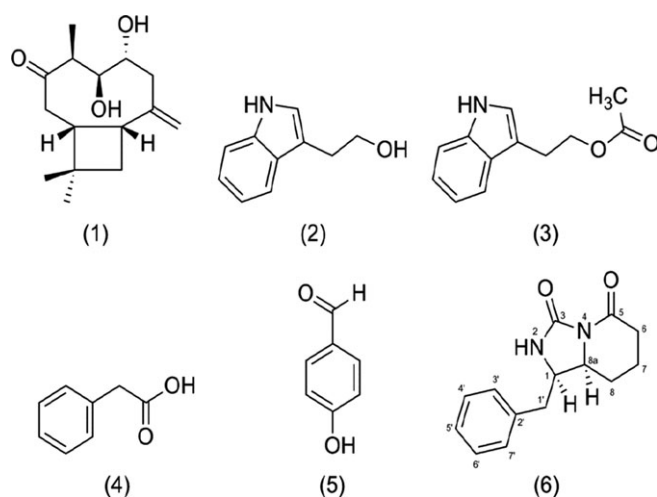


Fig. 1 Extrolites isolated from *Wallemia sebi* UAMH7897 filtrate extract: wallemione (1), tryptophol (2), tryptophol acetate (3), phenylacetic acid (4), *p*-hydroxybenzoic acid (5), and wallimidione (6)

Table 1 ^1H NMR Data (400 MHz, δ in ppm, J in Hz) and ^{13}C NMR Data (100 MHz, δ in ppm) for Wallimidione (6) in CD_3OD

(6)		
Position	δ_{C} , type	δ_{H} (J in Hz)
1	57.7, CH	4.44 t (5.1)
3	166.9, C	
5	170.0, C	
6	45.9, CH_2	3.37 m 3.53 dt (8.3, 12.0)
7	22.7, CH_2	1.75–1.83 m
8	29.4, CH_2	2.10 m 1.19 m
8a	60.0, CH	4.04 ddd (1.7, 6.5, 10.9)
1'	38.2, CH_2	3.16 dd (2.7, 5.1)
2'	137.3, C	
3'	131.0, CH	7.20–7.30 o
4'	129.4, CH	7.20–7.30 o
5'	128.1, CH	7.20–7.30 o
6'	129.4, CH	7.20–7.30 o
7'	131.0, CH	7.20–7.30 o

including two carbonyls and one aromatic carbon, five aromatic methines, two sp^3 methines, and four sp^3 methylenes.

Analysis of ^1H NMR spectroscopic data revealed the presence of a mono-substituted aromatic ring. In the HMBC spectrum, the cross-peak observed between the methylene protons at δ_{H} 3.16 (dd, J = 2.7, 5.1 Hz) and the aromatic carbons at δ_{C} 137.3 (quaternary), δ_{C} 129.4, and δ_{C} 131.0, located this methylene group at the benzylic position.

In the COSY NMR spectrum, an extended spin system was observed from the benzylic methylene protons at δ_{H} 3.16, sequentially to the two aliphatic methines at δ_{H} 4.44 (t, J = 5.1 Hz) and δ_{H} 4.04 (ddd, J = 1.7, 6.7, 10.9 Hz), to the three methylenes at δ_{H} 1.75–1.83 (m), δ_{H} 2.10, 1.19 (m), and δ_{H} 3.37 (m), 3.53 (dt, J = 8.0,

12.0 Hz). The terminal unequivalent methylene protons, H-6, in the spin system showed HMBC correlations with a carbonyl at δ_{C} 170.0. The two methines at δ_{H} 4.44 and δ_{H} 4.04 displayed HMBC correlations with carbonyls at δ_{C} 170.0 and 166.9, respectively. The carbon chemical shifts of the methines at δ_{C} 57.7 and 60.0 suggested that they are nitrogenated. It was deduced that the two nitrogens directly bound to the methines form amide bonds with the respective carbonyls at δ_{C} 170.0 and δ_{C} 166.9. The high-resolution mass and spectroscopic data indicated the presence of an imidazo [1,5- α] pyridine-3,5-dione substituted with a benzyl moiety. The structure of (6) was determined to be 1-benzylhexahydroimidazo [1,5- α] pyridine-3, 5-dione. The relative stereochemistry of positions H-1 and H-8a was determined by NOE. An NOE correlation was observed between H-1 and H-8a indicating that the two stereogenic methines are in the same plane.

Wallemione (1) was isolated as a colorless oil; $[\alpha]_{\text{D}}^{25} + 35$ (c 0.2, MeOH); UV (MeOH)/nm λ_{max} (log ϵ) 296 (2.20), 230 (2.56); HRESIMS m/z 253.1819 $[\text{M}+\text{H}]^+$ (calculated for $\text{C}_{15}\text{H}_{25}\text{O}_3$, 253.1804). ^1H NMR (CD_3OD , 400 MHz) and ^{13}C NMR (CD_3OD , 100 MHz) data were consistent with published data (Frank et al., 1999).

Wallimidione (6); light yellow oil; $[\alpha]_{\text{D}}^{25} - 68$ (c 0.2, MeOH); UV (MeOH)/nm λ_{max} (log ϵ) 275 (2.72), 248 (2.99) 220 (3.11); HRESIMS m/z 245.1293 $[\text{M}+\text{H}]^+$ (calculated for $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_2$, 245.1290); ^1H NMR (CD_3OD , 400 MHz); and ^{13}C NMR (CD_3OD , 100 MHz) data are reported in Table 1.

Antigen discovery

Indirect ELISA was performed as a preliminary means of assessing the reactivity between crude extracellular extracts of the six strains of *W. sebi* tested and the human polyclonal antibodies (HpAbs) in the sera from 84 atopic patients (Provost et al., 2013). Of these, 66 sera produced adequate response for subsequent immunoblotting to visualize proteins from culture extracts from each *W. sebi* strain. Optimal conditions were determined as 1 μg crude protein, HpAbs diluted 2000 times in 50% glycerol and 1% BSA-TBST, and alkaline phosphatase (AP)-conjugated mouse anti-human IgG diluted 10 000 times in 1% BSA/TBST. Major proteins common to all strains include proteins with approximate molecular weights of 97, 78, 64, 62, 52, 47, 41, and 31 kDa (Figure 2, Panel a).

Each immunoblot was analyzed, and the various reacting proteins were noted for each serum sample. The intensity of the protein bands on each immunoblot was graded on a scale of 0–3 (zero being no response, 1 being a weak response, 2 being a moderate response, and 3 being a strong response). Some human sera samples reacted with several proteins but showed lower responses in the corresponding indirect ELISA, while

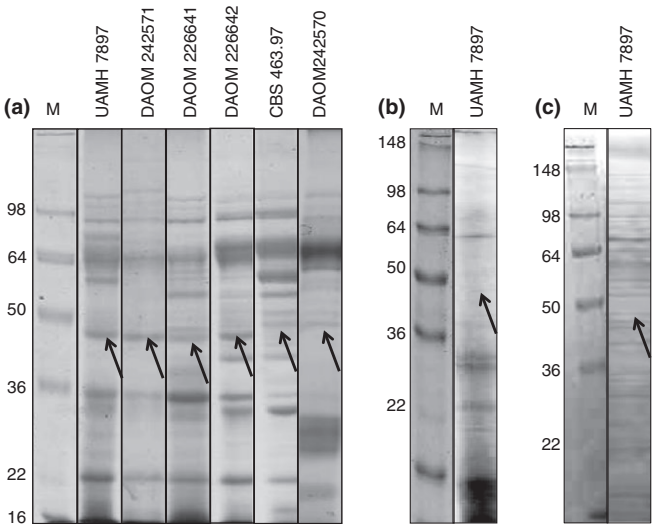


Fig. 2 SDS-PAGE of *Wallemia sebi* Coomassie stain illustrating extracellular proteins, intracellular proteins, and proteins found in arthrospores. Panel a shows the Coomassie Brilliant–Blue-stained gel of crude extracellular protein from several different indoor strains of *W. sebi*. Panel b shows the Coomassie Brilliant–Blue-stained gel of crude intracellular protein from *W. sebi* UAMH 7897. Panel c shows the Coomassie Brilliant–Blue-stained gel of crude arthrospore protein from *W. sebi* UAMH 7897

others gave a strong reaction in both the ELISA and corresponding immunoblot (Figure S1). Based on these data, the 47 kDa protein was chosen for further study. Additionally, this protein was observed in the extracellular proteins extracts of all six *W. sebi* strains studied. Figure 3 provides an example of strong immunoblot response against the 47 kDa protein. Human sera that

reacted with the 47 kDa sample are summarized in Table S1. This revealed that 36% of the patient sera tested responded to the antigen. The presence of the target protein in cells and spores was visualized by SDS-PAGE followed by CBB staining (Figure 2, Panels b and c), and the 47 kDa protein was also detected in spore extracts.

Further studies were performed with extracts of *W. sebi* UAMH 7897. The 47 kDa protein was purified from the crude extracellular protein extract using FPLC anion exchange chromatography. Approximately 350 mg of crude protein was loaded onto the column. The different proteins in the sample were separated and eluted using an increasing concentration of 1 M NaCl in 20 mM Tris buffer, pH 7.5. Aliquots of eluted fractions were separated by SDS-PAGE, and proteins were visualized by CBB staining allowing fractions containing the 47 kDa target protein to be identified.

Rabbit polyclonal antibodies produced to the target protein responded to the purified target protein as well as with the intracellular protein and spore protein extracts on Western immunoblots and with a capture ELISA (Figure S2). This confirmed the antigenicity of the target protein in rabbits in addition to humans and, importantly, the presence of high concentrations of the antigen in arthrospores.

Protein characterization

The 47 kDa protein had an isoelectric point (pI) of 4.9 and was glycosylated (Figure 4). The deglycosylation procedure revealed that the extent of glycosylation was

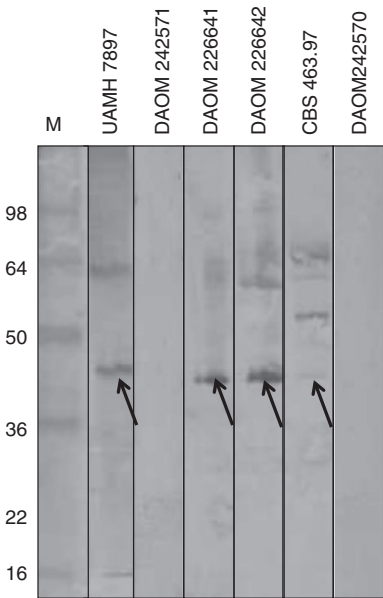


Fig. 3 Immunoblot of *Wallemia sebi* comparing the response of HpAb QC 3343 to crude extracellular proteins from different *W. sebi* strains. Arrows indicate 47 kDa protein

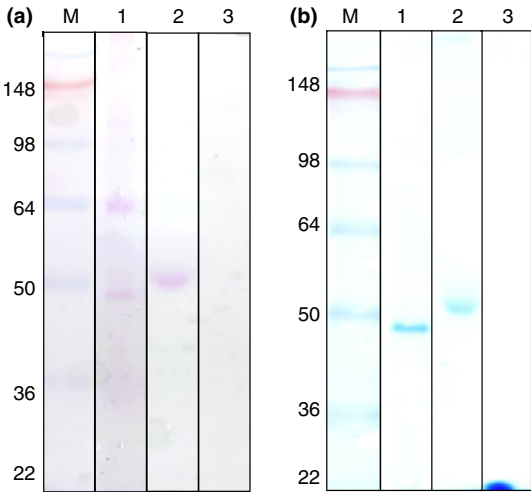


Fig. 4 Glycoprotein analysis of purified target protein. Panel a shows the SDS-PAGE gel stained with Coomassie Brilliant Blue. Panel b shows proteins stained with GelCode glycoprotein staining kit. Lane M: molecular weight marker; lane 1: purified target protein; lanes 2 and 3: horseradish peroxidase (positive glycoprotein control) and trypsin inhibitor (negative glycoprotein control), respectively

~5% by weight. The semipurified protein sample was separated by two-dimensional SDS-PAGE and visualized Western blot with enhanced anti-*W. sebi* rabbit polyclonal antibody. Proteins were applied to IPG strips with a pH range of 4–7 (7 cm) and left overnight to rehydrate. The IPG strips were focused for roughly 25 000 V h on a Protean IEF cell as described above and loaded onto two SDS-PAGE gels for separation in the second dimension. Proteins were transferred by immunoblot to a PVDF membrane and stained with IgG-specific antibodies to confirm the presence of the targeted antigenic protein on the first gel. The second gel was silver stained, and the corresponding protein was excised, digested with trypsin in-gel and analyzed by nano-scale liquid chromatographic tandem mass spectrometry (nLC-ESI-MS/MS; Table 2). A Mascot database search performed on the peptide MS/MS spectra identified a cellulase (gi|388581511) from *W. sebi* CBS633.66. When using a Mascot score >30, seven peptides were observed accounting for 24% sequence coverage of the full length protein. When setting the Mascot score to 20, the sequence coverage increased to 35% due to the addition of amino acids 171–213. This is most likely due to the proline at position 185 inducing conformational restriction in the peptide chain located directly after lysine 184, the only trypsin cleavage site between these amino acids. The experimentally determined pI 4.9 compared favorably with the predicted pI of 4.97. Based on analysis with SignalP 4.1, the antigenic cellulase contained a 23 amino acid signal peptide indicating this is an extracellular protein (Figure 5; underlined).

Discussion

There is strong epidemiological evidence from several countries that exposures to building/home dampness and mold have been associated with elevated risk of respiratory symptoms, asthma exacerbation, hypersensitivity pneumonitis, rhinosinusitis, bronchitis, and respiratory infections. The health effects observed are now thought to result from a mixture of allergens and various inflammatory compounds (Akpınar-Elci et al., 2013; Miller et al., 2010; Neveu et al., 2011; Rand et al., 2011, 2013).

Extrolites produced by strains of *W. sebi* from the built environment included the known compounds walleminone (Figure 1; **1**) and tryptophol (**2**). We did not observe walleminol by LC-UV-MS. Natural inter-conversion of walleminol to walleminone most likely occurs by an epoxidation of the *E*-alkene in walleminol, followed by a subsequent rearrangement to walleminone. This is known in the case of other naturally occurring caryophyllenes (Frank et al., 1999). This is the first report of tryptophol acetate (**3**) from this fungus. This was not unexpected as tryptophol (**2**) was reported as a major impurity during the isolation of walleminone (Frank et al., 1999). We can find no previous report of phenylacetic acid (**4**) and *p*-hydroxybenzoic acid (**5**) from *W. sebi*. Structurally similar para-substituted aromatic metabolites and other indole compounds were reported by Peng et al. (2011). We characterized a new metabolite, wallimidione (**6**). The structure of this compound appears to be rather unusual. No other natural products with the same

Table 2 Sequence fragments from the nLC-ESI-MS/MS analyses of the 47 kDa *Wallemia sebi* antigenic cellulase

Observed (<i>m/z</i>)	MR (expt.)	MR (calc.)	Error (Da)	Peptide	Sequence
434.2438 (2+)	866.4730	866.4722	0.0008	90–97	K.NAGLTHVR.I
1123.5829 (2+)	2245.1512	2245.1532	−0.0019	98–117	R.IPVGFWAIEQTGEPYIVGNR.L
1103.5541 (2+)	2205.0935	2205.0926	0.0009	135–155	K.VWIDLHGAPGSQNGLDNSGLR.T
920.9195 (2+)	1839.8244	1839.8248	−0.0004	156–170	R.TNNVQWHTDQNNVDR.S
819.8492 (2+)	1637.6838	1637.7256	−0.0418	353–366	R.QMWNTQVDAFEGGR.G
567.7496 (2+)	1133.4845	1133.5334	−0.0489	367–374	R.GYFFWTWK.N
684.7692 (2+)	1367.5238	1367.5742	−0.0503	375–385	K.NEEAADWSYQR.L

MKFNILALAA	AVAASPLQRR	YGDILIRGVNI	GGLFVLEPWI	TPSVFDQTGN	50
PAIVDEWTFG	QYQDHAQAES	AINSHLETFF	TYDDFQQIKN	AGLTHVRIPV	100
GFWAIETQGE	PYIVGNRLNK	LKEVVRWCRD	IGLK VWIDLH	GAPGSQNGLD	150
NSGLRTNNVQ	WHTDQNNVDR	SLSYIQTLD	EFTKPEYGAI	VEAIELLNPE	200
QSATHPEMLG	TLKSFYQNGY	GIVSQKAATA	IHDGFLDVNQ	WNDFLTSPQE	250
NVYLDTHKYQ	VFSDQQLQSS	DEQRTGAICQ	FKDKFAEHTA	NQHWVITGEW	300
SLATTDICARY	LNGRGIGARY	DGSYSGSSYV	GSCQKGTGDG	SDWSEELYKNQ	350
LR QMWNTQVD	AFEGGRGYFF	WTWKNEEAD	WSYQRLLQLG	IIPQDPNSYQ	400
HGICG					405

Fig. 5 Amino acid sequence of the 47 kDa antigenic cellulase isolated from *Wallemia sebi* UAMH7897. Underlined is the 23 amino acid signal peptide, and sequenced peptides are indicated in bold when utilizing a Mascot score >30

hexahydroimidazo [1,5- α] pyridine-3,5-dione core structure could be found in a substructure similarity search of the ACS database SciFinder®. An analysis for structural alerts of wallemine with TOXTREE (EU Joint Research Centre, Institute for Health and Consumer Protection) confirmed the available data showing modest toxicity. However, a similar analysis of the novel compound wallimidione revealed several indications of toxicity, requiring further testing.

IgG antibodies represent approximately 75% of circulating immunoglobulins and have a much longer half-life than IgE antibodies (Faquim-Mauro et al., 2003; Nissen et al., 1998). IgG and corresponding anti-IgG antibodies were chosen for recognition of fungal antigens. The first step in the antigen discovery involved screening crude protein extracts against the pre-screened human sera by immunoblotting and ELISA methods.

Using indirect ELISA, semipurified protein extracts examined responded to IgG antibodies in 66 of the 84 human sera tested. All six strains had a comparable average reactivity against all HpAbs screened. On immunoblot, major proteins were observed corresponding to molecular weights of 97, 78, 64, 62, 52, 47, 41, and 31 kDa by SDS-PAGE (Table S1). Approximately 40% of the 84 patient sera tested reacted strongly to the 47 kDa protein and was chosen for further work. The protein was purified from culture filtrate by a variety of techniques.

Wallemia sebi UAMH 7897 was chosen for the purification of the target protein based on its high-target protein yield, response to the 47 kDa protein on immunoblots and the total protein activity, as determined by ELISA. In this experiment, crude protein was extracted from the culture filtrate and concentrated 40 times using a concentrator equipped with a 10 000 molecular weight cut-off membrane. Approximately 90 mg of crude protein was obtained per l, representing approximately 3% of the total protein.

Rabbit polyclonal antibodies were produced by immunizing rabbits. A capture ELISA demonstrated that the purified rabbit antibodies responded to the 47 kDa protein was present in arthrospores and arthrospore-spiked dust. Capture ELISA was used to assess the cross-reactivity to proteins from fungi commonly found indoors, and none was observed. Evolutionary-close fungal species typically produce allergens that have a higher degree of similarity and that are likely to induce IgE cross-reactivity in exposed, sensitized individuals species (Soeria-Atmadja et al., 2010). Typically, a sequence identity of >50% is required for cross-reactivity to occur (Simon-Nobbe et al., 2007). These observations are consistent with what is known about the taxonomic position of this fungus.

We determined that the 47 kDa protein had an isoelectric point (pI) of 4.9 and was 5% glycosylated (w/w). There is a full sequence of *W. sebi* (Padamsee

et al., 2012) that facilitated the identification of the protein as a cellulase. At the time of writing, ca. 100 fungal allergens have been registered by the International Union of Immunological Societies (IUIS) (www.allergen.org). Of these, 82 allergens are from ascomycetes and 23 from basidiomycetes. The prevalence of sensitized individuals responding to the allergens we have studied in the sera from atopic patients used has generally accorded with the frequency of the species in damp buildings in the US/Canada. Approximately 10% of the patient sera responded to the *Stachybotrys chartarum sensu lato* antigen (Xu et al., 2007). In the case, *Aspergillus versicolor*, the value was 20% (Liang et al., 2011). The Fleming clade of *Penicillium chrysogenum* (now called *Penicillium rubens*; Houbraken et al., 2011) is the most common species of *Penicillium* on damp building materials Miller et al., 2008). For this species, just over 50% of the patient sera in our collection reacted to the allergen (Wilson et al., 2009). In the case of the very common *Chaetomium globosum*, approximately 50% of the sera tested responded to the antigen (Provost et al., 2013). For *W. sebi*, the value was 36%, further evidence of just how common sufficient exposures occur in damp buildings in North America.

In summary, we found that these strains of *W. sebi* isolated from the built environment in Canada produced a range of extrolites. These included the known compounds wallemine (1), tryptophol (2), tryptophol acetate (3), phenylacetic acid (4), and *p*-hydroxybenzoic acid (5) as well as the novel compound 1-benzylhexahydroimidazo [1,5- α] pyridine-3,5-dione which we have called wallimidione (6). In contrast to the known low toxicity of wallemine, *in silico* analysis of potential toxicity of the novel compound wallimidione gives several indications of toxicity. The 47 kDa excreted cellulase is present in high concentrations in arthrospores and is antigenic in humans and rabbits. This antigen is unrelated to previously reported allergens and is not present in extracts of other damp building fungi.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. A three-dimensional graph illustrating ELISA and immunoblot response of *W. sebi* strains against 31 individual patient sera.

Figure S2. Capture ELISA demonstrating the reactivity of the RpAbs from rabbit sera and *Wallemia sebi*

purified antigen (a), arthrospores (b) and arthrospore-spiked fine dust (c).

Table S1. Human sera ('QC' that gave a positive immunoblot response to the 47 kDa protein and other major *Wallemia sebi* proteins from various strains of *W. sebi*.

References

- Akpınar-Elci, M., White, S.K., Siegel, P.D., Park, J.H., Visotcky, A., Kreiss, K. and Cox-Ganser, J.M. (2013) Markers of upper airway inflammation associated with microbial exposure and symptoms in occupants of a water-damaged building. *Am. J. Ind. Med.*, **56**, 522–530.
- Amend, A., Seifert, K., Samson, R. and Bruns, T. (2010) Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics. *Proc. Natl. Acad. Sci. U S A*, **107**, 13748–13753.
- American Industrial Hygiene Association (AIHA) (2013) *Position Statement on Mold and Dampness in the Built Environment*, Fairfax, VA, American Industrial Hygiene Association <http://www.aiha.org/government-affairs/PositionStatements/P-Mold-03-26-13.pdf>
- Badar, Y., Lockley, W., Toubé, T., Weedon, B. and Guy-Valadon, L. (1972) Natural and synthetic pyrrol-2-ylpolyenes. *J. Chem. Soc., Perkin Trans. 1*, **13**, 1416–1424.
- Cramer, R. (2011) The problem of cross-reactivity in the diagnosis of fungal allergy. *Clin. Exp. Allergy*, **41**, 302–304.
- Faquim-Mauro, E., Jacysyn, J. and Macedo, M. (2003) Anaphylactic and non-anaphylactic murine IgG1 differ in their ability to bind to mast cells. *Immunobiology*, **207**, 169–177.
- Frank, M. and Hess, E. (1941) Studies on salt fish: V. Studies on *Sporendonema epizoom* from "Dun" salt fish. *J. Fish. Board Can.*, **5**, 276–286.
- Frank, M., Kingston, E., Jeffery, J.C., Moss, M.O., Murray, M., Simpson, T.J. and Sutherland, A. (1999) Wallemiol and wallemione, novel caryophyllenes from the toxigenic fungus *Wallemia sebi*. *Tetrahedron Lett.*, **40**, 133–136.
- Halsey, J.F., Jensen, J.T. and Miller, J.D. (2000) Immunological responses to *Stachybotrys chartarum*. *J. Allergy Clin. Immunol.*, **107**, 317.
- Health Canada (2007): *Residential Indoor Air Quality Guidelines: Moulds*, Ottawa, ON, Health Canada. Available at http://www.hc-sc.gc.ca/ewh-semt/alt_formats/hecs-sesc/pdf/pubs/air/mould-moisissures-eng.pdf
- Houbraken, J., Frisvad, J.C. and Samson, R.A. (2011) Fleming's penicillin producing strain is not *Penicillium chrysogenum* but *P. rubens*. *IMA Fungus*, **2**, 87–95.
- Ito, M., Tsukida, K. and Toubé, T.P. (1981) Pyrrolipolyenes. Part 3. Synthesis of all-(E)-wallemia C and stereochemistry of natural wallemia C. *J. Chem. Soc. Perkin. Trans.*, **1**, 3255–3257.
- Kolossa-Gehring, M., Becker, K., Conrad, A., Ludecke, A., Riedel, S., Seiwert, M. and Szwed, R. (2007) German environmental survey for children (GerES IV) - First results. *Int. J. Hyg. Environ. Health*, **210**, 535–540.
- Krieger, J., Jacobs, D.E., Ashley, P.J., Baeder, A., Chew, G.L., Dearborn, D., Hynes, H.P., Miller, J.D., Morley, R., Rabito, F. and Zeldin, D.C. (2010) Housing interventions and control of asthma-related indoor biologic agents: a review of the evidence. *J. Public Health Manag. Pract.*, **16**, s11–s20.
- Lappalainen, S., Pasanen, A., Reiman, M. and Kallioikoski, P. (1998) Serum IgG antibodies against *Wallemia sebi* and *Fusarium* species in Finnish farmers. *Ann. Allergy Asthma Immunol.*, **82**, 585–592.
- Liang, Y., Zhao, W., Xu, J. and Miller, J.D. (2011) Characterization of two related exoantigens from the biodeteriogenic fungus *Aspergillus versicolor*. *Int. Biodeterior. Biodegradation*, **65**, 217–226.
- Luo, W., Wilson, A.M. and Miller, J.D. (2010) Characterization of a 52 kDa exoantigen of *Penicillium chrysogenum* and monoclonal antibodies suitable for its detection. *Mycopathologia*, **169**, 15–26.
- Lustgraaf, B. (1978) Seasonal abundance of xerophilic fungi and house-dust mites (Acarida: Pyroglyphidae) in mattress dust. *Oecologia*, **36**, 81–91.
- Mendell, M.J., Mirer, A.G., Cheung, K., Tong, M. and Douwes, J. (2011) Respiratory and allergic health effects of dampness, mold, and dampness-related agents: a review of the epidemiologic evidence. *Environ. Health Perspect.*, **119**, 748–756.
- Miller, J.D. and Day, J.D. (1997) Indoor mold exposure: epidemiology, consequences and immunotherapy. *Can. J. Allergy & Clin. Immunol.*, **2**, 25–32.
- Miller, J.D., Rand, T., McGregor, H., Solomon, J. and Yang, C. (2008) Mold ecology: recovery of fungi from certain moldy building materials. In: Prezant, B., Weekes, D. and Miller, J.D. (eds) *Recognition, Evaluation and Control of Indoor Mold*, Fairfax, VA, American Industrial Hygiene Association, 43–51.
- Miller, J.D., Sun, M., Gilyan, A., Roy, J. and Rand, T.G. (2010) Inflammation-associated gene transcription and expression in mouse lungs induced by low molecular weight compounds from fungi from the built environment. *Chem. Biol. Interact.*, **183**, 113–124.
- National Institute for Occupational Safety and Health (NIOSH) (2012) *Preventing Occupational Respiratory Disease from Exposures Caused by Dampness in Office Buildings, Schools, and Other Nonindustrial Buildings*, Cincinnati, OH, NIOSH Publication # 2013–102. National Institute for Occupational Safety and Health (www.cdc.gov/niosh).
- Neveu, W.A., Bernardo, E., Allard, J.L., Nagaleekar, V., Wargo, M.J., Davis, R.J., Iwakura, Y., Whittaker, L.A. and Rincon, M. (2011) Fungal allergen b-glucans trigger p38 mitogen-activated protein kinase-mediated IL-6 translation in lung epithelial cells. *Am. J. Respir. Cell Mol. Biol.*, **45**, 1133–1141.
- Nissen, D., Peterser, L., Esch, R., Svejgaard, E., Skov, P. and Poulsen, L. (1998) IgE-sensitization to cellular and culture filtrates of fungal extracts in patients with atopic dermatitis. *Ann. Allergy Asthma Immunol.*, **81**, 247–255.
- Nonnenmann, M., Coronado, G., Thompson, B., Griffith, W., Hanson, J., Vesper, S. and Faustman, E. (2012) Utilizing pyrosequencing and quantitative PCR to characterize fungal populations among house dust samples. *J. Environ. Monit.*, **14**, 2038–2043.
- Padamsee, M., Kumar, T.K., Riley, R., Binder, M., Boyd, A., Calvo, A.M., Furukawa, K., Hesse, C., Hohmann, S., James, T.Y., LaButti, K., Lapidus, A., Lindquist, E., Lucas, S., Miller, K., Shantappa, S., Grigoriev, I.V., Hibbett, D.S., McLaughlin, D.J., Spatafora, J.W. and Aime, M.C. (2012) The genome of the

- xerotolerant mold *Wallemia sebi* reveals adaptations to osmotic stress and suggests cryptic sexual reproduction, *Fungal Genet. Biol.*, **49**, 217–226.
- Peng, X.P., Wang, L., Liu, P.P., Hong, K., Chen, H., Yin, X. and Zhu, W.M. (2011) Aromatic compounds from the halotolerant fungal strain of *Wallemia sebi* PXP-89 in a hypersaline medium, *Arch. Pharm. Res.*, **34**, 907–912.
- Provost, N., Shi, C., She, Y., Cyr, T.D. and Miller, J.D. (2013) Characterization of an antigenic chitosanase from the cellulolytic fungus *Chaetomium globosum*, *Med. Mycol.*, **51**, 290–299.
- Quansah, R., Jaakkola, M.S., Hugg, T.T., Heikkinen, S.A. and Jaakkola, J.J. (2012) Residential dampness and molds and the risk of developing asthma: a systematic review and meta-analysis, *PLoS One*, **11**, e47526.
- Rand, T.G., DiPenta, J., Robbins, C. and Miller, J.D. (2011) Inflammation associated gene modulation in mouse alveolar macrophages induced by low molecular weight compounds from fungi associated with damp building environments, *Chem. Biol. Interact.*, **190**, 139–147.
- Rand, T.G., Robbins, C., Rajaraman, D., Sun, M. and Miller, J.D. (2013) Induction of Dectin-1 and asthma-associated signal transduction pathways in RAW 264.7 cells by a triple-helical (1, 3)- β -D glucan, curdlan, *Arch. Toxicol.*, **87**, 1841–1850.
- Reboux, G., Piarroux, R., Mauny, F., Madroszyk, A., Millon, L., Bardonnnet, K. and Dalphin, J.C. (2001) Role of molds in farmer's lung disease in Eastern France, *Am. J. Respir. Crit. Care Med.*, **163**, 1534–1539.
- Sakamoto, T., Urisu, A., Yamada, M., Matsuda, Y., Tanaka, K. and Torii, S. (1989) Studies on the osmophilic fungus *Wallemia sebi* as an allergen evaluated by skin prick test and radioallergosorbent test, *Int. Arch. Allergy Appl. Immunol.*, **90**, 368–372.
- Samson, R.A., Houbaken, J., Thrane, U., Frisvad, J.C. and Andersen, B. (2010) *Food and Indoor Fungi. CBS Laboratory Manual, Series 2*, Utrecht, the Netherlands, CBS-Fungal Biodiversity Centre
- Simon-Nobbe, B., Denk, U., Pöll, V., Rid, R. and Breitenbach, M. (2007) The spectrum of fungal allergy, *Int. Archives Allergy Immunol.*, **145**, 58–86.
- Soeria-Atmadja, D., Onell, A. and Borgå, A. (2010) IgE sensitization to fungi mirrors fungal phylogenetic systematics, *J. Allergy Clin. Immunol.*, **125**, 1379–1386.
- Takahashi, T. (1997) Airborne fungal colony-forming units in outdoor and indoor environments in Yokohama, Japan, *Mycopathologia*, **139**, 23–33.
- Takahashi, I., Maruta, R., Ando, K., Yoshida, M., Iwasaki, T., Kanazawa, J., Okabe, M. and Tamaoki, T. (1993) UCA1064-B, a new antitumor antibiotic isolated from *Wallemia sebi*: production, isolation and structural determination, *J. Antibiot. (Tokyo)*, **46**, 1312–1314.
- Twine, S., Mykytczuk, N., Petit, M., Tremblay, T., Conlan, W. and Kelly, J. (2005) *Francisella tularensis* proteome: low levels of ASB-14 facilitate the visualization of membrane proteins in total protein extracts, *J. Proteome Res.*, **4**, 1848–1854.
- Wheeler, K.A., Hocking, A.D. and Pitt, J.I. (1988) Effects of temperature and water activity on germination and growth of *Wallemia sebi*, *Trans. British Mycol. Soc.*, **90**, 365–368.
- Wilson, A.M., Luo, W. and Miller, J.D. (2009) Using human sera to identify a 52 kDa exoantigen of *Penicillium chrysogenum* and implications of polyphasic taxonomy of anamorphic ascomycetes in the study of allergens, *Mycopathologia*, **168**, 213–226.
- Wood, G., Mann, P.L., Reid, W. and Moss, M. (1990) Studies on a toxic metabolite from the mould *Wallemia*, *Food Addit. Contam.*, **7**, 69–77.
- World Health Organization (WHO) (2009) *WHO Guidelines for Indoor Air Quality: Dampness and Mould*, Copenhagen, Denmark, World Health Organization, Regional Office for Europe, DK-2100.
- Xu, J., Jensen, J.T., Liang, Y., Belisle, D. and Miller, J.D. (2007) The biology and immunogenicity of a 34 kDa antigen of *Stachybotrys chartarum sensu lato*, *Int. Biodeg. Biodec.*, **60**, 308–318.
- Zalar, P., de Hoog, G.S., Schroers, H.J., Frank, J.M. and Gunde-Cimerman, N. (2005) Taxonomy and phylogeny of the xerophilic genus *Wallemia* (Wallemiomycetes and Wallemiales, cl. et ord. nov.). *Antonie Van Leeuwenhoek*, **87**, 311–328.