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Secondary metabolites from *Eurotium* species, *Aspergillus calidoustus* and *A. insuetus* common in Canadian homes with a review of their chemistry and biological activities

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ABSTRACT

As part of studies of metabolites from fungi common in the built environment in Canadian homes, we investigated metabolites from strains of three *Eurotium* species, namely *E. herbariorum*, *E. amstelodami*, and *E. rubrum* as well as a number of isolates provisionally identified as *Aspergillus ustus*. The latter have been recently assigned as the new species *A. insuetus* and *A. calidoustus*. *E. amstelodami* produced neoechinulin A and neoechinulin B, epiheveadride, flavoglaucin, auroglaucin, and isotetrahydroauroglaucin as major metabolites. Minor metabolites included echinulin, preechinulin and neoechinulin E. *E. rubrum* produced all of these metabolites, but epiheveadride was detected as a minor metabolite. *E. herbariorum* produced cladosporin as a major metabolite, in addition to those found in *E. amstelodami*. This species also produced questin and neoechinulin E as minor metabolites. This is the first report of epiheveadride occurring as a natural product, and the first nonadride isolated from *Eurotium* species. Unlike strains from mainly infection-related samples, largely from Europe, neither ophiobolins G and H nor austins were detected in the Canadian strains of *A. insuetus* and *A. calidoustus* tested, all of which had been reported from the latter species. TMC-120 A, B, C and a sesquiterpene drimane are reported with certainty for the first time from indoor isolates, as well as two novel related methyl isoquinoline alkaloids.

Introduction

The growth of fungi or mould on wet or chronically damp building materials or contents is associated with increased exacerbation of asthma in people with mould allergy and with increased risk of respiratory disease (NAS 2000; 2004; Health Canada 2007: http://www.hc-sc.gc.ca/ewh-semt/alt_formats/hecs-sesc/pdf/pubs/air/mould-moisissures-eng.pdf). Above a certain threshold, living or working in a building derived with mould and dampness problems results in exposure to spores and hyphal fragments. A higher percentage of the smaller particles get deeper in the lung than the larger materials, such as spores. All these materials contain allergens/antigens, beta 1, 3 D-glucan

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and species-specific low molecular weight metabolites (Green *et al*. 2006).

We have been investigating the antigens and toxins of fungi that are common in damp buildings in Canada and the USA. For both types of work, reliable taxonomy is critical, including in those instances where there are species recognizable by molecular information but for which there are no accepted morphospecies. An example of this is the four clades of *Penicillium chrysogenum*. Only one of these is common in house dust, and the ''indoor clade'' makes particular metabolites (Scott *et al*. 2004; De La Campa *et al*. 2007). This enabled us to screen indoor strains for proteins antigenic in humans from this clade (Wilson 2008), as well as to investigate the relevant metabolites in rodent lung cell models (Rand *et al*. 2005, 2006).

In North America, *Eurotium herbariorum* and, to a lesser extent, *E. rubrum* and *E. amstelodami*, are common on mould-damaged, paper-faced gypsum wallboard, manufactured wood, ceiling tiles, insulation, and textiles, which have been somewhat wet or subject to periodic condensation (Flannigan & Miller 2001; Miller *et al*. 2008). These *Eurotium* species are found in grain products, poultry feed, bakery products, dried fruits, spices, soil, hypersaline waters, and Dead Sea soil (Butinar *et al*. 2005). *Aspergillus* section *Usti* has been recently studied resulting in the description of *A. insuetus* and *A. calidoustus* (Houbraken *et al*. 2007; Varga *et al*. 2008). These two species grow well at 37 °C, whereas *A. ustus* does not. *A. ustus s. lat*. is common on mould-damaged, paper-faced gypsum wallboard, manufactured wood and insulation (Miller *et al*. 2008). Notably for species that are common on wallboard and some other building materials, salt tolerance is a critical physiological trait. It might be anticipated that the fungi that grow on wallboard must also be tolerant of calcium salts. Based on their recovery from salty/alkaline soils, strains of *E. herbariorum* (among other species that appear on wallboard) were tolerant to very high concentrations of calcium in soils (Butinar *et al*. 2005; Steiman *et al*. 1997) and are extremely xerophilic (Flannigan & Miller 2001). *A. ustus s. lat*. is also moderately salt tolerant, but is hydrophilic (minimum $a_w \geq 0.9$). From the limited data available, *A. calidoustus* was reported from indoor air, raw rubber, wood from the built environment, and *A. insuetus* is probably a soil fungus. *A. ustus s. lat*. has been long regarded as a facultative pathogen, but these strains have been referred to *A. calidoustus* and, apparently, to a lesser extent, *A. insuetus* (Houbraken *et al*. 2007; Varga *et al*. 2008). *A. ustus s. lat*. has been regarded as an uncommon soil fungus, which is apparently the niche of *A. ustus*. Based on sparse data, all three species are reported from indoor air (Houbraken *et al*. 2007).

Making use of cultures collected from some commercial laboratories in Canada, we examined the metabolites produced by a number of poorly-studied fungi that are common from samples from indoor air-quality surveys and some isolated from mouldy building materials. Some are from outdoor air samples taken adjacent to the test site. The species tested were *Eurotium amstelodami*, and, to a lesser extent, *E. rubrum* and *E herbariorum*. Strains of *A. insuetus* and *A. calidoustus* were also studied. These were formerly described as *A. ustus* and are common on mouldy building materials in North America.

Materials and methods

Cultures

Isolates of strains of *Eurotium amstelodami*, *E. herbariorum*, *E. rubrum,* and *Aspergillus ustus s. lat*. were collected from air samples taken in or near buildings in Canada (Ontario, Manitoba, Saskatchewan, and Alberta). Less than half of the *E. amstelodami* isolates came from reference air samples taken adjacent to the building, with the remainder isolated from indoor air samples or directly from mould-damaged materials. Isolates of the remaining species were all from indoor air samples. Additional *A. ustus s. lat*. strains were studied but not deposited in IBT or CBS because either they were unproductive or their metabolite patterns were similar (Table 2).

The isolates were studied morphologically following the recommended method and media (Samson *et al*. 2004), and their identity was confirmed using the analysis of the beta tubulin sequences as described by Houbraken *et al*. (2007). All isolates of *Eurotium* and *Aspergillus* showed to be typical representatives of the species.

Fermentation and metabolite screening

Several isolates of each species were tested in media that had been reported from the literature to be useful for metabolite production in these fungi. Single spore isolates were made and cultured on slants containing 2 % malt extract (ME; Difco Laboratories, Detroit, MI) agar. A slant was macerated in sterile water. The resulting suspension was used to inoculate three Roux bottles [5 % (v/v)] containing 200 ml sterile Czapek–Dox supplemented with yeast extract (CY medium; 30 g l^{-1} sucrose, 1 g l^{-1} K_2HPO_4, 5 g l^{-1} yeast extract (Difco), 0.5 g l^{-1} $MgSO_4$, 3 g l^{-1} $NaNO_3$, 0.01 g l^{-1} $FeSO_4$, 0.5 g l^{-1} KCl) or 2 % ME. The cultures were incubated at 25 °C for two weeks in the dark.

After two weeks of growth, the cultures were filtered by suction using Whatman no. 1 filter paper, and the volume and pH of the recovered filtrates were measured before they were extracted twice with equal volumes of ethyl acetate (EtOAc) in a large separation funnel. The combined organic layers were filtered (Whatman no. 1) through anhydrous Na_2S0_4, concentrated under vacuum, and dried under nitrogen gas to afford crude filtrate extracts. The mycelium was wrapped in aluminium foil, frozen, and freeze-dried. The mycelium was weighed, ground, and extracted with EtOAc (250 ml) with constant stirring overnight. The solution was filtered and processed as previously described for the filtrate, yielding mycelial extracts.

The crude filtrate and mycelial extracts for all species were initially screened for metabolite production by tlc (0.2 mm silica gel 60 F_{254} pre-coated alumina) using an optimally determined 10 % methanol–chloroform ($MeOH–CHCl_3$) solvent system. They were viewed under UV light (UVP Chromto-Vue C-70 G) by both short wave (254 nm) and long wave (365 nm) light. The plates were then dipped in a molybdenum solution [90 ml H_2O, 10 ml H_2SO_4, 2.5 ml ammonium molybdate, 1 g cerium (IV) sulphate] and dried with a heat gun until dark spots appeared in visible light where compounds were present.