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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A B S T R A C T

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 isothetrahydroauroglaucin 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.

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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...
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 aw > 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 K₂HPO₄, 5 g l⁻¹ yeast extract (Difco), 0.5 g l⁻¹ MgSO₄, 3 g l⁻¹ NaNO₃, 0.01 g l⁻¹ FeSO₄, 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 Na₂SO₄, 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 F₂₅₄ pre-coated alumina) using an optimally determined 10 % methanol–chloroform (MeOH–CHCl₃) solvent system. They were viewed under UV light (UVF 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 H₂O₂, 10 ml H₂SO₄, 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.
A single method of screening was developed for hplc-DAD analysis using a Hewlett Packard Agilent 1100 series quaternary pump with a photodiode array detector (Agilent, Mississauga, ON connected to a Phenomenex Synergi Max-RP C12 (250 x 4.6 mm, 4 µm, 80 Å) column (Torrance, CA). A gradient mobile phase of acetonitrile-water (CH$_3$CN–H$_2$O) with trifluoroacetic acid (TFA); [0.05 % (v/v)] with a 1 ml min$^{-1}$ flow rate was used. It employed a gradient beginning at 0 % CH$_3$CN, increasing to 100 % CH$_3$CN over a 23 min time period before returning to the starting conditions for the remaining 2 min. The detector was set at 210 nm and 254 nm for compound detection. All extracts were dissolved in CH$_3$CN and filtered through syringe filters (Puradisc, 0.22 µm membrane; Maidstone, UK) prior to injection.

**Isolation and purification of metabolites**

In order to achieve quantities of metabolite necessary for structural elucidation, large-scale fermentations of productive strains were performed. Eurotiurn amstelodami (IBT 28307), E. herbariorum (CBS 47-F8), and Aspergillus calidoustus (IBT 28269, IBT 28272) were chosen based on tlc and hplc-DAD analysis. They were fermented in CY medium using 10 l Glaxo bottles for each of these strains. Purification of all Eurotiurn metabolites was accomplished by prep-tlc (Partisil PK5F, 150 Å, 1000 mm) using a 10 % MeOH–CHCL$_3$ solvent system. Isolated metabolites were further purified using a Waters Delta Prep 3000 prep-hplc system (Millford, MA) with a Waters 484 tunable absorbance detector connected to a Phenomenex Synergi Max-RP C12 (250 x 21.2 mm, 10 µm, 80 Å) column and employing the same gradient change as in the screening process, but increasing the solvent flow to 25 ml min$^{-1}$. A. calidoustus metabolites were crudely purified using solid-phase extraction columns (Strata-x-33 µm polymeric RP, Phenomenex) with compounds eluting between 0–50 % CH$_3$CN–H$_2$O. Additional purification was achieved using prep-hplc (as above) but employing a CH$_3$CN–H$_2$O gradient (10–90 % CH$_3$CN) over a 40 min period with a flow rate of 25 ml min$^{-1}$. All resulting metabolite suspensions were dried by vacuum and stored in glass amber vials. Metabolite purity was tested by a combination of hplc-DAD, nuclear magnetic resonance (NMR), mass spectrometry (MS), and various spectral data in comparison to literature values. The resulting purified metabolites were used as standards.

**NMR, LC-MS and IR analysis**

Metabolite spectra were acquired on a Bruker Avance 300 spectrometer (Milton, ON) at 300.13 MHz ($^1$H) or 75 MHz ($^{13}$C), using a 5 mm inverse triple (H/C/N) probe. When required, compounds were analysed with a Bruker Avance 400 MHz with 5 mm auto-tuning broadband probe with a Z gradient.

**Table 1 – Metabolites from various Canadian strains of Eurotiurn amstelodami, E. herbariorum, and E. rubrum fermented on CY (µg g$^{-1}$ mycelium$^{-1}$)**

<table>
<thead>
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<th>Strain</th>
<th>Filtrate</th>
<th>Mycelium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Neoechinulin B</td>
</tr>
<tr>
<td>Eurotiurn amstelodami</td>
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<td></td>
</tr>
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</tr>
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<tr>
<td><em>Mean</em></td>
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<td>1700 ± 310</td>
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<tr>
<td>E. herbariorum</td>
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<td></td>
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<tr>
<td>CBS 47-F3</td>
<td>651</td>
<td>1795</td>
</tr>
<tr>
<td>CBS 47-F8</td>
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<tr>
<td>CBS 47-G1</td>
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<td>–</td>
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<tr>
<td><em>Mean</em></td>
<td>570 ± 95</td>
<td>1206 ± 398</td>
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<td>E. rubrum</td>
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<td>CBS 47-F4</td>
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<td>7963</td>
</tr>
<tr>
<td>CBS 47-F6</td>
<td>680</td>
<td>637</td>
</tr>
<tr>
<td><em>Mean</em></td>
<td>1140 ± 412</td>
<td>3720 ± 2193</td>
</tr>
</tbody>
</table>

~ Below limit of detection.

a Mean of positives ± s.e.
Spectra were determined in CD$_3$CN, CDCl$_3$, or C$_3$D$_6$O and chemical shifts were reported relative to tetramethylsilane (TMS). Chemical shift assignments were made with $^1$H/$^1$H correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY), $^1$H/$^{13}$C heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and distortionless enhancement by polarization transfer (DEPT-135) inverse correlation spectra using standard Bruker pulse sequences. High-resolution mass spectrometry (HRMS) data was obtained on a Kratos Concept instrument. FT-IR (Fourier transform infrared spectroscopy) (Varian 1000 Scimitar Series; Missisauga, ON) analysis was performed on all major compounds. LC-MS analysis was performed according to Nielsen & Smedsgaard (2003) for determination of known minor metabolites.

Recovery studies

For quantitative analysis, a 1 mg ml$^{-1}$ stock solution of each compound (when available) was made up in CH$_3$CN to create a range of concentrations for standard curve construction. The standard curve was obtained by plotting hplc-DAD peak area counts versus amount of compound loaded on the column at 254 nm. Concentrations of 1, 0.1, and 0.01 mg ml$^{-1}$, representing 10 to 0.1 µg of the compound on the column were used. From the standard curve and the percent recovery of the extraction procedure, the production of each metabolite by each strain could be quantified in (µg g$^{-1}$ l$^{-1}$). For recovery studies, varying quantities of isolated major metabolites were used to spike 50 ml sterile CY medium in triplicate. Quantities representing 5, 10, and 20 µg were used. The spiked media was extracted, dried, and analysed by hplc-DAD as before. Peak identities were confirmed by comparing resulting spectra to the standard UV full-scan spectra and recoveries determined. Quantitative data are presented as micrograms per gram of mycelium per litre to accommodate differences in the growth rates of the fungi (see also De La Campa et al. 2007).

Results

Eurotium

Cultures of several Eurotium amstelodami strains grown on CY medium yielded more biomass (× 16) than on 2 % ME. E. herbariorum and E. rubrum did not grow on ME; CY medium was used thereafter. Indoor and outdoor strains of E. amstelodami and E. rubrum produced indistinguishable chemical profiles in the filtrate extract regardless of environment. Analysis of E. herbariorum filtrate extracts revealed the production of those major metabolites detected in the above Eurotium species, as well as an additional major metabolite. Analysis of the mycelial extracts of all three species was qualitatively similar.

Major metabolites of E. amstelodami and E. rubrum filtrate extracts (Table 1) were identified as neoechinulin A (Fig 1A) and neoechinulin B (Fig 1B). Epheveadride (Fig 1C) was identified as a major metabolite in E. amstelodami, but as a minor metabolite in E. rubrum. E. herbariorum filtrate extracts contained those found in E. amstelodami, as well as cladosporin (Fig 1D) and apparent cladosporin-like derivatives based on similar $^1$H NMR and UV absorbances to that of cladosporin. Mycelial extracts for all three species contained flavoglaucin (Fig 1E), auroglaucin (Fig 1F) and isotetrahydroauroglaucin

![Fig 1 - Secondary metabolites detected from Eurotium species (* detected in the mycelium, † minor metabolites).](image-url)
Echinulin (Fig 1H), preechinulin (Fig 1I), and neoechinulin E (Fig 1J) were detected as minor metabolites in the culture extracts of *E. amstelodami* and *E. rubrum*, whereas questin (Fig 1L and Fig 1K) were detected in that of *E. herbariorum*. Identifications were based on known molecular masses and comparison with published data (Nielsen & Smedsgaard 2003). Metabolite structures are presented in Fig 1. In general, all the major metabolites were produced by the strains tested, except for cladosporin, which was only produced by *E. herbariorum*. Higher quantities of flavoglaucin, auroglaucin, and isotetrahydroauroglaucin were detected in mycelial extracts; whereas in culture filtrates, neoechinulins A and B and epiheveadride were found in highest quantities. In contrast to the other strains, epiheveadride was a minor compound in the *E. rubrum* strains tested (Table 1).

The analytical method used for *Eurotium* compounds was satisfactory over the required concentration ranges. For both neoechinulin A and B, a linear relationship with an $R^2$ value of 1 was found over the full concentration range. The other compounds measured had similar values, i.e. epiheveadride $R^2 = 0.99$ and cladosporin $R^2 = 0.99$. Recovery from spiked medium was determined for neoechinulin A (82.6 % ± 2.5), neoechinulin B (84.0 % ± 3.7), epiheveadride (85.4 % ± 2.5) and cladosporin (87.6 % ± 2.0). The limits of detection averaged ca 1 µg ml$^{-1}$ and the limit of quantification >2.5 µg ml$^{-1}$.

**Aspergillus insuetus and A. calidoustus**

All strains of these species grew well in CY medium (15.12 ± 1.68 g l$^{-1}$), but poorly to nil in ME media. There were no significant differences in mycelial mass among the strains on CY medium. Major metabolites were identified almost exclusively from the filtrate extract as TMC-120 A (Fig 2A), TMC-120 C (Fig 2C), novel TMC-120 derivative 1 (Fig 2D), TMC-120 derivative 2 (Fig 2E) and a drimane sesquiterpene (Fig 2F). These known and novel TMC-120 isoquinoline alkaloids were detected primarily in *Aspergillus calidoustus* strains, whereas only in one strain of *A. insuetus* (Table 2). The identification of TMC-120 B (Fig 2B) by LC-MS analysis was based on published UV and molecular mass data (Kohno et al. 1999a). Metabolite structures are presented in Fig 2. Previously reported compounds such as ophiobolin G, ophiobolin H, and austin by Cutler et al. (1984), using potato–dextrose agar (PDA) media, and by Ahmed et al. (1989) using ME liquid culture, were not detected by LC-MS analysis of these extracts against authentic standards.

The analytical method used for the *Aspergillus* compounds was satisfactory over the required concentration ranges. For TMC-120 A and B and TMC-120 novel derivative 1, a linear relationship with an $R^2$ value of 1 was found over the full concentration range. The other compounds measured had similar values, i.e. compound TMC-120 C $R^2 = 0.98$; compounds TMC-120 novel derivative 2 and drimane sesquiterpene $R^2 = 0.99$. Recovery from spiked medium was determined for TMC-120 A and B (79.4 % ± 2.6), TMC-120 C (79.7 % ± 3.5), TMC-120 novel derivative 1 (83.4 % ± 2.4), TMC-120 novel derivative 2 (77.5 % ± 4.6), and drimane sesquiterpene (75.3 % ± 3.7). The limits of detection averaged 1 µg ml$^{-1}$ and the limit of quantification, >2.5 µg ml$^{-1}$.

Fig 2 – Secondary metabolites detected from the crude filtrate extracts of *A. insuetus* and *A. calidoustus.*
Table 2 – Metabolites from various Canadian strains of Aspergillus insuetus and *A. calidoustus* fermented on CY (μg g⁻¹ mycelium 1⁻³⁻³⁻³⁻³)²

<table>
<thead>
<tr>
<th>Strain</th>
<th>TMC-120 A</th>
<th>TMC-120 C</th>
<th>TMC-120 derivative 1</th>
<th>TMC-120 derivative 2</th>
<th>Drimane</th>
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<tr>
<td><em>Mean</em></td>
<td>186</td>
<td>146 ± 38</td>
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<tr>
<td><em>Mean</em></td>
<td>173 ± 64</td>
<td>142 ± 38</td>
<td>13 ± 3</td>
<td>52 ± 29</td>
<td>50 ± 24</td>
</tr>
</tbody>
</table>

–, Below the limit of detection. a Mean of positives.

Metabolite chemical data

Unless otherwise stated, chemical and NMR data are reported in Slack (2007). The remaining data presents a record of the purity of the material isolated for toxicity testing (neoechinulin A, B, c ladosporin, TMC-120 A and B) or as comparison to the new metabolites reported.

Eurotium amstelodami, *E. herbariorum* and *E. rubrum*

Neoechinulin A was crystallized as ivory crystals from methanol, melting point (m.p.) 262–264 °C, with UV maxima at: 225 (100), 288 (50), and 233 (55) by hplc-DAD. The optical rotation was [α]D²⁵ = 50° (c = 1, CHCl₃), in agreement with literature values (Aoki et al. 2006). The mass spectrum displayed [M + 1]⁺ at: m/z 324, and HRMS established the molecular formula, C₁₉H₂₁N₃O₂, (calculated 323.1647, found 323.1628). The NMR data were consistent with published data (Dossena et al. 1974). Neoechinulin B was crystallized as yellow crystals from methanol, m.p. 235–237 °C, with UV maxima at: 229 (100), 272 (66), and 368 (32) by hplc-DAD. The mass spectrum showed [M + 1]⁺ at: m/z 322 and HRMS established the molecular formula, C₁₉H₂₁N₃O₂ (calculated 321.1476, found 321.1477). MS (EI) m/z (rel. int.): 252 ([M]⁺ – 69 or C₆H₅/isoprene unit, 100). These data were consistent with Marchelli et al. (1977).

Epiheveadride MS data accorded with Crane et al. (1973) and NMR data were consistent with Hosoe et al. (2004a).

Cladosporin was crystallized from methanol as pale yellow crystals, m.p. 193–194 °C, with UV maxima at: 218 (100), 269 (62), and 302 (24) by hplc-DAD. The optical rotation was determined at: [α]D²⁵ = 15° (c = 1, MeOH). The mass spectrum displayed [M + 1]⁺ at m/z 293 and HRMS established the molecular formula, C₁₉H₂₀O₅, (found 292.1305, calculated 292.1305). MS (EI) m/z (rel. int.): 292 ([M]⁺, 14), 193 (7), 99 (100). The spectral data were consistent with published data (Jacyno et al. 1993).

Flavoglaucin chemical data were in agreement with Allen et al. (1978); auroglaucin and isotetrahydroauroglaucin with Hamasaki et al. (1980); preechinulin, neoechinulin E, and echinulin with Marchelli et al. (1977); Nielsen & Smedsgaard (2003) and questin with Choi et al. (2007).

Aspergillus insuetus and *A. calidoustus*

TMC-120 A was isolated as a pale yellow solid, m.p. 117–118 °C, with UV maxima at: 218 (100), 240 (83), and 365 (32) by hplc-DAD. The optical rotation was determined at: [α]D²⁵ = 15° (c = 1, CHCl₃) and HRMS established the molecular formula of C₁₅H₁₅NO₂, (found 241.1101, calculated 241.1097). MS (EI) m/z (rel. int.): 241 ([M]⁺, 79), 226 (98), 199 (100), 185 (99). Both the ¹H NMR and ¹³C NMR were consistent with literature values (Kohno et al. 1999a).

TMC-120 B and TMC-120 C data were consistent with Kohno et al. (1999a). A sesquiterpene drimane was isolated and a detailed analyses of the COSY, HSQC, and DEPT-135 spectra compared with referenced data of drim-7-en-11,12-oxide (Hayes et al. 1995).

Novel TMC-120 derivatives

The diagnostic spectral features observed for the novel methyl isouquinoline alkaloids TMC-120 derivatives 1 and 2 resembled those previously reported for TMC-120 A-C (Kohno et al. 1999a; Ogawa et al. 2004). Their fluorescent characteristics were also comparable with those known for isouquinoline pigments panafollines A–D (Kinoshita et al. 2003, 2005). The absolute
stereochemistry of TMC-120 derivatives 1 and 2 was deduced from the NOESY experimental data for TMC-120 B reduced products (Kohno et al. 1999b) and X-ray crystallography on panaefluoroline D (Kinoshita et al. 2005). NMR data are reported in Table 3 and compared with known TMC-120 alkaloids A–C.

TMC-120 novel derivative 1 was isolated as a fluorescent blue/green amorphous solid with UV maxima at: 210 (100), 247 (75), 313 (8), and 464 (12) by hplc-DAD. The optical rotation revealed a molecular formula of C15H17NO2 (found: m/z (rel. int.): 243 (75), 313 (8), and 464 (12) by hplc-DAD. The optical rotation TMC-120 products, new resonances in the 1H NMR spectrum revealed a molecular formula of C15H17NO2 (found: m/z (rel. int.): 243 (61), 184 (100), 172, (48). The base peak at m/z 184 was attributed to bond cleavage between C-2 and C-1, leading to the loss of (CH3)2COH. IR peak at m/z 184 was attributed to bond cleavage between C-

The 13C NMR and HSQC data established the methyl isoquinoline structure of TMC-120 C between C-2 and C-3 bonds. IR$_{max}$ (CHCl$_3$, cm$^{-1}$) exhibited major bands for OH (3197), NH (2976, 2927), aromatic (1594, 1571), and ether (1273). In comparison to known TMC-120 products, new resonances in the 1H NMR spectrum were assigned to the oxygenated quarternary carbon, and at δ 90.8 (C-2), 30.4 (C-3), 24.1 (C-12), and 23.3 (C-13) accounted for the remaining part of the molecule. The proposed structure was established by strong HMBC cross-peaks from H-3 to C-2, C-4, C-9b, and C-11; and from H-12 and H-13 (the two methyl singlets) to C-2 and C-11.

TMC-120 novel derivative 2 was isolated as a yellow amorphous solid with UV maxima at: 202 (100), 218 (93), 245 (86), 260 (82), and 360 (39) by hplc-DAD. The optical rotation was determined at: [α]$^\text{D}$ +32$^\pm$32$^\circ$ (c = 1, MeOH) and HRMS established the formula of C$_{15}$H$_{17}$NO$_2$ (found: m/z 184, calculated: 259.1180). MS (EI) m/z (rel. int.): 259 ([M$^+$], 17), 186 (100). The base peak at m/z 186 indicated the loss of C$_4$H$_9$O from a cleavage of the C-2 and C-3 bonds. IR$_{max}$ (CHCl$_3$, cm$^{-1}$): 3195 (OH), 2927 (NH), 1260 (ether). This novel product contained two deuterium oxide (D$_2$O) exchangeable singlets at δ 13.56 (δ = 13.56, 1H) and δ 13.56 (δ = 13.56, 1H) confirmed this deduction. The 13C NMR and HSQC data displayed a ketone carbonyl at 205.6 (C-3) and an oxygenated methine at δ 76.5 (C-2). The proposed structure was verified by strong HMBC cross-peaks from H-2 and the two methyl groups at δ 20.3 (C-12) and at δ 14.6 (C-13); between H-4 and the ketonic carbon C-3, C-3a, C-5a and C-9b, and between H-11 with the two methyl groups C-12 and C-13. This novel TMC-120 derivative may be a product from ring opening of TMC-120 C between C-2 and the oxygen at position 1.

**Discussion**

As noted, the three Eurotium species as studied are common on many mould-damaged building materials in the US and Canada. The majority of isolates were from air samples with some that were obtained directly of building materials. The strains of Aspergillus insuetus and A. calidoustus tested are the first reports of these taxa from the built environment in Canadian buildings noting that A. ustus s. lat. was common on a shorter list of mould-damaged building materials (Miller et al. 2008). Regardless of the new compounds reported here, this study represents

<table>
<thead>
<tr>
<th>Position</th>
<th>TMC-120A</th>
<th>TMC-120C</th>
<th>TMC-120 derivative 1</th>
<th>TMC-120 derivative 2</th>
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<tr>
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<td>δC</td>
<td>δH (J, Hz)</td>
<td>δC</td>
</tr>
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<td>197.4</td>
<td>3.35 (d, 9.0, 2H)</td>
<td>30.4</td>
</tr>
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<td>115.4</td>
<td>120.2</td>
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<tr>
<td>4</td>
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<tr>
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<tr>
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<tr>
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<td>5.1</td>
<td>3.46 (br s, 1H)</td>
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</table>
the first detailed chemical studies of the metabolites from these fungi in most cases since their first reports. For compounds where there were differences with literature values and/or in relation to the novel compounds, modern spectroscopic data are reported and clarified, and quantities were isolated and are documented for toxicology studies.

These built-environment strains of three Eurotium species produced a common metabolite profile, all producing large amounts of flavoglaucin, auroglaucin, and isosterotetrahydorauroglaucin (Fig 1E–G) as well neoechinulins A and B (Fig 1A–B) echinulin, preechinulin, neochinulin E (Fig 1H–J) epeheveadride (Fig 1C) and questin (Fig 1K). Differences included that E. herbariorum produced cladosporin (Fig 1D) and much reduced yield of epeheveadride in the E. rubrum strains tested. The diketopiperazine alkaloids and epeheveadride were not detected in mycelium, but were found in the culture filtrate. Flavoglaucin, auroglaucin, and isosterotetrahydorauroglaucin were found only in the mycelial extracts. Growths of unidentified species of Eurotium on wood and manufactured woods incubated under different conditions were physically removed and analysed using high-resolution mass spectrometry. The resulting extracts contained fewer compounds but were otherwise similar (Nielsen et al. 2004). This is the first report of epeheveadride as a natural product, the first monadride discovered in Eurotium, and of cladosporin from E. herbariorum from the built environment.

Eurotium was originally known for producing hydroquinone pigments and a variety of anthraquinones, such as physcion and questin (Anke et al. 1980; Gould & Raistrick 1994). These have been reported to occur mainly in the mycelium of a number of Eurotium species (Nazar et al. 1984; Hamsaki et al. 1980). Questin has been isolated from many Aspergillus species (Grove 1973; Liu et al. 2006), as well as Chrysosporium species (Slater et al. 1971), and Microascus tardifaciens (Fujimoto et al. 1999).

Echinulin was first discovered in E. echinulatum (Quilico et al. 1949) and has been reported from various strains of E. amstelodami (Allen 1972; Gatti & Fuganti 1979), E. repens (Smetanina et al. 2007). Neoecchinulin A first appeared in the literature as a co-metabolite of neoecchinulin B and other neochinulins (C–E) in E. amstelodami (Dossena et al. 1974; Marchelli et al. 1977) and with neoecchinulin in E. amstelodami (Marchelli et al. 1977).

As reported in the present study, flavoglaucin and auroglaucin co-occur in various Eurotium species and along with isosterotetrahydorauroglaucin in some strains. Chemotypes of the Eurotium species in the present work were consistent with isolates from food, salt soils among other habitats. Cladosporin also known as asperentin, was originally discovered in C. cladosporioides (Scott et al. 1971; Jacyno et al. 1993). E. repens has been reported as a cladosporin producer (Butinar et al. 2005; Smetanina et al. 2007). In contrast, we found that cladosporin was produced only by E. herbariorum. As our report is consistent with studies done mainly with European strains, our data support the views that chemotypes of these cosmopolitan fungi are independent of region of origin.

Epheveadride is the first monadride discovered in Eurotium and this is the first report of this compound occurring as a natural product. It was originally reported as a synthetic product and a diastereomer of heveadride, a fungal metabolite in Helminthosporium heveae (Hosoe et al. 2004b; Crane et al. 1973). It exhibited strong antifungal activity against A. fumigatus, Penicillium marneffei, Trichophyton rubrum, and T. mentagrophytes (Hosoe et al. 2004a).

The toxicity of echinulin was studied by Ali et al. (1989) in female mixed-breed rabbits by intraperitoneal (i.p.) injection of 10 mg kg⁻¹ BW purified compound. Pathological studies of the organs revealed severe damage of alveolar organization and thickening of alveolar walls and liver damage. Echinulin was cytotoxic to HeLa cells at 100 µg ml⁻¹ (Umeda et al. 1974) but not genotoxic by the hepatocyte primary culture/DNA repair test using hepatocytes isolated from ACI rat and C3H/HeN mice livers at 10⁻⁴ µ (Mori et al. 1984). Feed refusal in swine at 8 µg g⁻¹ was attributed to echinulin in the diet and in Sprague–Dawley female and male mice (five to seven weeks old) were used for a drinking water bioassay. Mice also refused to drink water containing 90 µg ml⁻¹ echinulin (Vesonder et al. 1988).

Neoechinulin has been shown to exhibit cytoprotection activity against peroxynitrite-induced PC12 cell death (Kuniaki et al. 2007) and SIN-1-induced cell death have been documented (Kiyotoshi et al. 2004). It also demonstrated a higher anti-oxidant activity than tocopherol (Ryuta & Mikiharu 1999), and radical scavenging activity against diphenylpicryl hydrazyl (DPPH) (Yong et al. 2004).

Flavoglaucin has been reported as weakly cytotoxic to HeLa cells (Umeda et al. 1974); to cause hepatic damage in rabbits (Nazar et al. 1984); was negative in an HPC/DNA repair assay in rat and mouse hepatocytes (Mori et al. 1984); inhibited mitochondrial respiration, induced mitochondrial swelling (Kawai et al. 1986); and was Ames Test positive (Hamasaki et al. 1980). It demonstrated antioxidant activity and was reported as synergistic with tocopherol (Ishikawa et al. 1984). Both flavoglaucin and isosterotetrahydorauroglaucin exhibited radical scavenging activity against DPPH (Yong et al. 2006).

Cladosporin has insecticidal (Grove & Pople 1981), as well as antibacterial and antifungal, activity (Podojil et al. 1978). It also inhibited uracil and leucine uptake into Bacillus brevis cells (Anke et al. 1978), and the growth of etiolated wheat coleoptiles (Springer et al. 1981). Questin has antibiotic activity and inhibited nucleoside import by a tumour cell line and in mouse lymphocytes (Su et al. 2004).

Based mainly on strains isolated from diseased human tissue in Europe, A. insuetus, A. calidoustus and the closely related A. pseudodefectus, produced similar secondary metabolites (Houbraken et al. 2007; Varga et al. 2008). All produced drimanes, both A. calidoustus and A. insuetus produce ophiobolins G and H, whereas only A. calidoustus produced austins, compared with pergulin-like metabolites and many unknown metabolites biosynthesized by A. insuetus and A. pseudodefectus, respectively. Additionally, Nielsen (2003) suggested that TMC-120-related compounds were present in A. ustus var. pseudodefectus (revised as A. insuetus, pers. comm.) from LC-MS analysis of building-derived isolates. Nielsen also reported that the building-derived strains of A. ustus grown on chipboard and gypsum (with and without wallpaper) produced konatins and what were suggested as ophiobolins-type compounds (Nielsen 2003; Nielsen et al. 1999). It is likely that these strains are A. insuetus or A. calidoustus. The secondary metabolite profiles of A. insuetus and A. calidoustus originating from in Canada were similar to those of strains from the environmental and pathogenic strains of Houbraken et al. (2007) with some compounds missing and some new compounds produced.
found. Ophiobolins, Austin, and pergillin-like compounds were not detected under the conditions tested. In contrast, the strains we studied produced the methyl isoquinoline alkaloids, TMC-120-related compounds. Their identification from these newly circumscribed species in built-environments, are reported with certainty for the first time in this study. The discovery of the two novel TMC-120 derivatives may aid in chemically characterizing \textit{A. insuetus} and \textit{A. calidoustus}. The common occurrence of drimanes indicates a shared biosynthetic pathway with the mainly European pathogenic strains. Although most of the major and some minor chromatogram peaks of \textit{A. insuetus} and \textit{A. calidoustus} were assigned in this investigation, additional peaks still remained unknown (Slack 2007).

The metabolite profile differences in \textit{A. insuetus} and \textit{A. calidoustus} noted between these strains and previous reports may be due to geographic variation as for \textit{F. graminearum} (Miller et al. 1991), variation in chemotypes within strains of the same species as is the case in \textit{Stachybotrys chartarum} (Andersen et al. 2002) or variation of the response under different culture conditions. The conditions used were based on literature reports of the first discoveries of the metabolites. Studies comparing the metabolite patterns of some penicillia revealed modest differences between results from agar media, such as FDA and yeast extract–sucrose (YES), and ultimately metabolites were isolated on liquid cultures (Nielsen et al. 2006).

The TMC-120s were first reported as secondary metabolites of \textit{A. ustus} (TC 1118) isolated from rhizosphere of grass (Kohno et al. 1999a, 1999b). TMC-120 B and ester derivatives of the drimane have been isolated from \textit{A. pseudodefectus} (Ogawa et al. 2004), and \textit{A. ustus var. pseudodefectus} (Hayes et al. 1995), respectively. This lends support to the current view of the biochemical relatedness between these species. There are few toxicity data for the TMC-120 compounds, but they have been implicated as moderate inhibitors of eosinophil function and as possible anti-inflammatory agents (Kohno et al. 1999b). TMC-120 B did not inhibit cancer cell growth or the DNA polymerases tested (Ogawa et al. 2004). The endothelin binding activity of the drimane has been reported, together with its ester derivatives, which exhibited endothelin receptor binding inhibitory activity against rabbit endothelin receptors with IC\textsubscript{50} values between 20 - 150 \textmu M (Hayes et al. 1995).

In conclusion, \textit{E. herbariorum} and \textit{A. ustus} s. lat. are common biodeteriogens of a variety of building materials in North America. Based on pan-Canadian collections, we have clarified that the majority of strains reported as \textit{A. ustus} are \textit{A. insuetus} and \textit{A. calidoustus}. For the first time, North American strains from the built environment have been demonstrated to produce the characteristic metabolites of the species of \textit{Eurotium} studied. Additionally, \textit{E. herbariorum} was found to produce epi-heveadride, the first report of this compound occurring as a natural product, and the first nonadride isolated from \textit{Eurotium} species. Canadian strains of \textit{A. calidoustus} from the built environment are consistent producers TMC-120 A, B, C, and a sesquiterpene drimane. These are reported with certainty for the first time from this species as two novel, related methyl isoquinoline alkaloids. Across all strains, \textit{A. insuetus} produced TMC-120 A, B, C, albeit inconsistently, but the drimane was the consistently produced. Neither ophiobolins G and H, nor austins were detected under the conditions used.

As noted, the fungi studied are common on damp building materials in North America (Miller et al. 2008) and their metabolites had been previously studied mainly from food or medical samples from Europe. Previous studies had illuminated chemotype differences between strains of various species in fungi collected in the Americas versus those from Europe and Asia (Miller et al. 1991; Nielsen et al. 2006). The present work has resolved this question for the \textit{Eurotium} and Aspergillus species tested and permits toxicology studies to be undertaken similar to those described in Rand et al. (2005, 2006)

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References


