CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY OF THREE TANZANIAN WILD MUSHROOM SPECIES

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ABSTRACT

The three Tanzanian wild mushroom species Termitomyces letestui, Lactarius edulis and Agaricus sp. aff. arvensis yielded ergosterol, 5,8-peroxyergosterol and ergosta-5,22-dien-3β-ol, and a mixture of ergosterol, ergosta-7,22-dien-3β-ol and ergosta-7-en-3β-ol whose composition was deduced from gas chromatography/mass spectroscopic (GC/MS) analysis of the trimethylsilylated mixture. GC/MS analysis of the lipid fraction from T. letestui revealed the presence of linoleic (C₁₈:₂), stearic (C₁₈:₀), oleic (C₁₈:₁), palmitic (C₁₆:₀), pentadecanoic (C₁₅:₀) and myristic (C₁₄:₀) acid. Polar T. letestui and L. edulis fractions yielded α,α1,1′-trehalose and mannitol. Some of the crude extracts from the three mushroom species showed mild antimicrobial, mosquito larvicidal and cytotoxic activities. The chemical composition and antimicrobial activities infer that the three mushroom species are potential functional food substrates.

INTRODUCTION

Termitomyces is a tropical edible mushroom genus that occurs in symbiosis with termites (Harkonen et al. 2003). T. letestui is one of the Termitomyces species growing in Tanzania and usually appears at the onset of rains. Among the few reported chemical analyses of Termitomyces species include the recent isolation of neurotogenic cerebrosides from the edible Chinese mushroom Jizong [T. albuminusus (Berk.) Heim.] (Qi et al. 2000). So far, there are no reports in either chemical constituents or biological activity of T. letestui and this prompted us to include this mushroom species in our ongoing chemical analysis of Tanzanian wild mushrooms for bioactive and nutritional constituents (Mdachi et al. 2004).

Other Tanzanian wild mushrooms Lactarius eduli (Russulaceae), and Agaricus sp. aff. arvensis (Agaricale; Harkonen et al., 2003), that grow around decayed wood and termite hills in coastal areas of Tanzania, were also selected for our investigations, owing to their mild activity in the brine shrimp test (Meyer et al. 1982). Agaricus compounds include amino acids, steroids and physiologically active phenylhydrzones of glutamic acid (Chulia et al. 1988, Kawakishi et al. 1998).

In Africa, the mostly edible Lactarius species grow wildly in miombo woodlands, their colour and taste being associated with fatty acid esters metabolized as a response to injury (Bernardi et al. 1992, Sterner and Anke 1995, Stadler and Sterner, 1998). In a recent study, 15 out of 27 Tanzanian Lactarius species were for the first time scientifically described (Karhula et al. 1998), indicating the great abundance of Lactarius species in Tanzania.
MATERIALS AND METHODS

General experimental procedures
Column chromatography: Silica gel 60 (0.063 - 0.200 mm, Merck), gradient elution (petrol ether/EtOAc); TLC: Silica gel 60 F₂₅₄ (Merck) pre-coated on Al or plastic plates; visualization: UV-VIS and anisaldehyde (Stahl, 1969); recrystallization: Petrol ether/EtOAc (9:1 v/v) or MeOH; IR: Bruker IFS 28; specific rotation: Perkin Elmer Model 341 polarimeter; H and ¹³C NMR spectra: Varian Unity 300 or Bruker Avance DPX 300 at 300 MHz for ¹H and 75 MHz for ¹³C NMR, or Varian Inova 500 or Bruker Avance DRX 600 at 500 or 600 MHz for ¹H NMR, inverse techniques for HMOC and HMBC; chemical shifts in ppm [internal standard TMS (δ = 0 ppm) for ¹H and CDCl₃ (δ = 77.0 ppm) for ¹³C NMR]; high resolution negative ion ESI MS: Bruker Apex III FT Ion Cyclotron Resonance (FT-ICR) MS with an Infinity™ cell, a 7.0 T superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source; sample solutions introduced continuously via a syringe pump, flow rate of 120 µl/h.

Mushroom materials
Fruiting bodies of L. edulis and T. letestui were collected from Mafinga region in Tanzania (February – April 2003 and 2004) and authenticated at the Department of Molecular Biology and Biotechnology, University of Dar es Salaam where voucher specimens are preserved. Agaricus sp. aff. arvensis was collected from the University of Dar es Salaam main campus (April – May 2003 and 2004). The fruiting bodies immediately after collection were first oven dried (40 °C) for one day and then carefully dried at room temperature (25 °C) for one week.

Extraction and isolation
Dried, ground fruiting bodies of T. letestui (403 g), L. edulis (200 g) and Agaricus sp. aff. arvensis (203 g) were soaked in petrol ether, dichloromethane and EtOH or MeOH. A MeOH solution of the T. letestui EtOH extract (32 g) on cooling in the fridge at -4°C formed white crystals of mannitol and column chromatography of the concentrated filtrate yielded ergosterol, a mixture of free fatty acids (GC-MS), mannitol and α,α-1,1’-trehalose (2). Vacuum liquid chromatography (VLC) of the Lactarius edulis MeOH extract (10 g) followed by recrystallization yielded ergosta-5,22-dien-3β-ol, while the more polar VLC factions on crystallization in MeOH gave mannitol. The 2nd VLC fraction of the Agaricus sp. aff. arvensis EtOH extract (4.38 g) consisted of a mixture of ergosterol, ergosta-7,22-dien-3β-ol and ergosta-7-en-3β-ol and upon filtration the supernatant solution became pure ergosterol. Recrystallization of VLC fraction 3 (petrol ether/EtOAc, 9:1 v/v) yielded white crystals of compound 1. The rest of the polar fractions contained mixtures of fluorescing compounds that could not be separated even by reversed phase HPLC.

GC-MS analysis
The fatty acid composition of the lipid fraction from T. letestui as well as that of the trimethylsilylation mixture of sterols from Agaricus sp. aff. arvensis extracts product was determined by coupled, temperature programmed GC-MS analysis using an MD 800 GC-MS System (Fisons Instrument). The MS of each individual compound was compared with those contained in a computerized database.

Biological assays
Brine shrimp test, antimicrobial and mosquito larvicidal assays were carried out as described in the literature (Meyer et al. 1982, Moshi et al. 2004 and Joseph et al. 2004).

RESULTS
The L. edulis petrol ether, dichloromethane and MeOH extracts, and T. letestui EtOH extract exhibited mild cytotoxic activity (brine shrimp test – BST, LC₅₀ = 88, 69.6, 26.7 and 69.7 µg/ml respectively). The
Agaricus sp. aff. arvensis EtOH extract had the highest cytotoxic activity (LC\textsubscript{50} = 19.9 \mu g/ml) and as such it was also evaluated for lethality against An. gambiae mosquito larvae, whereby it exhibited mild activity (LC\textsubscript{50} = 0.52, 0.18 and 0.15 mg/ml after 24, 48 and 72 h exposure).

The crude dichloromethane and EtOH extracts from T. leestui and Agaricus sp. aff. arvensis exhibited moderate activity against the bacteria Vibrio cholerae and Escherichia coli, and the fungus Candida albicans (Table 1), while Agaricus sp. aff. arvensis EtOH extract exhibited high activity against the bacterium Bacillus anthracis, and mild activity against Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae and Proteus sp. (Table 1). Ergosterol showed weak activity against Bacillus anthracis and V. cholerae.

Table 1: Antimicrobial activity of some crude Agaricus sp aff arvensis and T. leestui extracts and egesterol

<table>
<thead>
<tr>
<th>Organism</th>
<th>Zones of inhibition (mm)</th>
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<tbody>
<tr>
<td></td>
<td>\textit{Agaricus sp.}(EtOH)</td>
</tr>
<tr>
<td>\textit{Escherichia coli}</td>
<td>6.5 ± 0.16</td>
</tr>
<tr>
<td>\textit{Salmonella boydii}</td>
<td>0</td>
</tr>
<tr>
<td>\textit{Salmonella typhimurium}</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>\textit{Klebsiella pneumoniae}</td>
<td>7 ± 0.14</td>
</tr>
<tr>
<td>\textit{Bacillus anthracis}</td>
<td>24 ± 0.2</td>
</tr>
<tr>
<td>\textit{Staphylococcus aureus}</td>
<td>10 ± 0.1</td>
</tr>
<tr>
<td>\textit{Vibrio cholerae}</td>
<td>10 ± 0.1</td>
</tr>
<tr>
<td>\textit{Proteus sp.}</td>
<td>8 ± 0.3</td>
</tr>
<tr>
<td>\textit{Candida albicans}</td>
<td>6.5 ± 0.16</td>
</tr>
</tbody>
</table>

Repeated chromatography of the EtOH or MeOH extracts from \textit{T. leestui}, \textit{L. edulis} and \textit{Agaricus sp.} aff. \textit{arvensis} yielded ergosterol, 5α,8α-epidioxy-ergost-6,22-dien-3β-ol (5,8-peroxyergosterol, 1), ergosta-5,22-dien-3β-ol and an inseparable mixture of ergosterol, ergosta-7,22-dien-3β-ol and ergosta-7-en-3β-ol as established from GC-MS analysis of the trimethylsilylated mixture.
5α,8α-Epidioxy-ergost-6,22-dien-3β-ol (5,8-peroxyergosterol) (1) exhibited the following properties: White crystals; m.p. 170 - 171°C, m.p. 178 - 179°C (Kocor and Szalowska 1972); yield, 7 mg; \( R_f = 0.5 \) (20% EtOAc/hexane); anisaldehyde – blue; EI-MS, m/z (% rel. int.) 400 ([M – C\(_2\)H\(_6\)]\(^+\), 35), 398 (40), 363 (25), 271 (100), 255 (80), 229 (30), 213 (30) and 147 (35); ESI-FT-ICR-MS (positive ion mode), m/z 451.31871 ([M + Na]+), calc. for \( \text{C}_{23}\text{H}_{33}\text{O}_{6}\text{Na} = 365.1054326 \); \(^1\)H NMR, \( \delta \) 3.45 (2H, dd, \( J = 9.2, 3.9 \) Hz, H-4 and H-4'), 3.64 (2H, dd, \( J = 9.9, 3.9 \) Hz, H-2 and H-2'), 3.76 (2H, dd, \( J = 11.9, 5.2 \) Hz, H-6p' and H-6b'), 3.84 (2H, m, H-5 and H-5'), 3.87 (2H, m, H-3 and H-3'), 3.88 (m, H-6c and H-6c'), 5.19 (2H, d, \( J = 3.9 \) Hz, H-1 and H-1') and \(^13\)C NMR, \( \delta \) 93.36 (C-1, C-1'), 72.69 (C-3, C-3'), 72.33 (C-5, C-5'), 71.22 (C-2, C-2'), 70.98 (C-4, C-4') and 60.71 (C-6, C-6').

**DISCUSSION**

Structure I was established based on \(^1\)H- and \(^13\)C-NMR data (Mekkawy et al. 1998), particularly H/H (COSY) and H/C (HMQC and HMBC) interactions and positive ion ESI-FT-ICR MS. The presence of the endoperoxide moiety was deduced from the low field position of the H-6 and H-7 resonances and appearance of \(^13\)C NMR resonances at \( \delta \) 82.12 and 79.39 that were attributed to the oxygenated quaternary carbon atoms 5 and 8, since all spectra indicated absence of acetylenic moieties that would have otherwise accounted for the latter signals. Many previous reports have suggested that peroxyergosterol may be an artifact rather than a true natural product (Nam et al. 2001). However, in these investigations the compound was detected even on TLC analysis of freshly obtained crude extracts, suggesting that the compound was indeed a true natural product. 5,8-Peroxyergosterol was previously reported as an antitumour compound (Kocor and Szalowska 1972, Brown and Jacobs 1975).

In previous studies, ergosterol derivatives exhibited antitumor, cytotoxic, rheumatoid arthritis and immune promoting properties (Brown and Jacobs 1975, Bod et al. 1999, Wasser and Weis 1999), the latter activity being a suitable attribute for food supplements. Therefore, due to the recently
increasing demand for food supplements (functional foods), particularly for individuals with compromised immunity, the mushroom species 

_Agaricus_ sp. aff. _arvensis_ and _L. edulis_, and other Tanzanian wild mushroom species (Nyigo _et al._ 2005) that are rich in the above steroids, are interesting candidates for evaluation as potential functional food substrates.

_Termotomyces lenestui_ polar fractions yielded \( \alpha,\alpha 1,1' \)-trehalose (2) whose structure was deduced from \(^1\text{H-}_{}\) and \(^{13}\text{C-}_{}\)NMR spectral data (Breitmair and Voelter 1987, Duddeck _et al._ 1998), [\( \alpha \)]\(_{\text{D}}\) and m.p. values (Matsuura _et al._ 2002) and positive ion ESI-FT-ICR MS that showed the molecular formula \( \text{C}_{12}\text{H}_{22}\text{O}_{11} \) for the disaccharide. The H/H and C/H interactions as observed in the COSY, HMQC and HMBC spectra revealed all the CHO connectivities and this unambiguously established the monosaccharide unit in structure 2. The MS exhibited a weak fragment ion peak at \( \text{m/z} 325 \) resulting from cleavage of a hydroxyl unit from the molecular ion, and another peak at \( \text{m/z} 163 \) due to a fragment ion for one of the two sugar residues. This and the fact that there was only one set of six signals in the \(^{13}\text{C-}_{}\) NMR spectrum indicated that the isolated compound was a symmetrical disaccharide.

\( \alpha,\alpha 1,1' \)-Trehalose which is hereby being reported for the first time in a _Termotomyces_ species was previously shown to possess \( \alpha \)-glucosidase inhibition, exhibiting ability to suppress postprandial hyperglycemia caused by prolonged high blood glucose levels associated with diabetes (Moordian and Thurman 1999, Matsuura _et al._ 2002), and hence indicating the potential nutritional value of _T. lenestui_ for individuals susceptible to diabetes.

GC/MS analysis of the combined less polar fractions from _T. lenestui_ indicated the presence of the free fatty acids linoleic (C\(_{18:2}\)), stearic (C\(_{18:0}\)), oleic (C\(_{18:1}\)), palmitic (C\(_{16:0}\)), pentadecanoic (C\(_{15:0}\)) and myristic (C\(_{14:0}\)) acid, as it was previously observed for other saprophytes, in comparison with symbiotrophs (Feofilova, 1998), thus further indicating that _T. lenestui_ possesses suitable attributes of a functional food substrate, since essential fatty acids are required for the promotion of a variety of body biochemical functions (Arasmus 1995).

It was surprising to note that the antimicrobial activity of the _Agaricus_ sp. aff. _arvensis_ EtOH extract was lost upon fractionation, indicating that either the activity was due to a combination of all the constituent compounds in the crude extract, or that the active compound(s) were labile, hence having been readily transformed into inactive products during fractionation.

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