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 trimethysilylated mixture. GC/MS analysis of the lipid fraction from* T. letestu *revealed the presence of 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. 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 neuritogenic cerebrosides from the edible Chinese mushroom *Jizong* [*T. albuminosus* (Berk.) Heim.] (Qi *et al*. 2000). So far, there are no reports on 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 phenylhydrazones 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 F_{254} (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; ^{1}H and ^{13}C NMR spectra: Varian Unity 300 or Bruker Avance DPX 300 at 300 MHz for ^{1}H and 75 MHz for ^{13}C NMR, or Varian Inova 500 or Bruker Avance DRX 600 at 500 or 600 MHz for ^{1}H NMR, inverse techniques for HMQC and HMBC; chemical shifts in ppm [internal standard TMS (δ = 0 ppm) for ^{1}H and $CDCl_3$ (δ = 77.0 ppm) for ^{13}C 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 district, Iringa 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 ^{O}C) for one day and then carefully dried at room temperature (25 ^{O}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 $\alpha,\alpha 1,1'$-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 on column chromatography and then recrystallization gave 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, LC_{50} = 88, 69.6, 26.7 and 69.7 µg/ml respectively). The

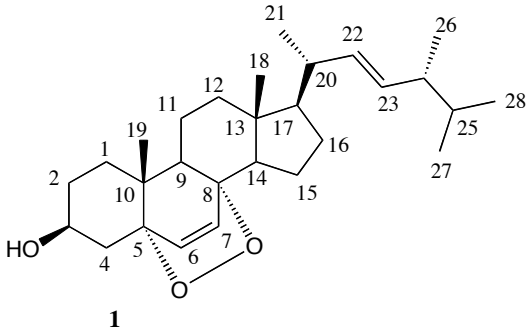
Agaricus sp. aff. *arvensis* EtOH extract had the highest cytotoxic activity (LC_{50} = 19.9 μg/ml) and as such it was also evaluated for lethality against *An. gambiae* mosquito larvae, whereby it exhibited mild activity (LC_{50} = 0.52, 0.18 and 0.15 mg/ml after 24, 48 and 72 h exposure).

The crude dichloromethane and EtOH extracts from *T. letestui* 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 *Basillus anthracis*, and mild activity against *Salmonella typhimurium, Staphylococcus aureus, Klebsiella pneumoniae* and *Proteus* sp. (Table 1). Ergosterol showed weak activity against *Basillus anthracis* and *V. cholerae.*

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

Organism	Zones of inhibition (mm)				
	Agaricus sp.(EtOH)	Ergosterol	*T. letestui* (CH_2Cl_2)	Ampicillin	Miconazole
Escherichia coli	6.5 ± 0.16	0	11 ± 0.6	26 ± 0.2	-
Salmonella boydii	0	0	0	18 ± 0.1	-
Salmonella typhimurium	11 ± 0.5	0	0	25 ± 0.2	-
Klebsiella pneumoniae	7 ± 0.14	0	0	20 ± 0.5	-
Basillus anthracis	24 ± 0.2	8.2 ± 0.2	0	27 ± 0.2	-
Staphylococcus aureus	10 ± 0.1	0	0	30 ± 0.1	-
Vibrio cholerae	10 ± 0.1	6.5 ± 0.5	10 ± 0.3	17 ± 0.4	-
Proteus sp.	8 ± 0.3	0	0	15 ± 0.5	-
Candida albicans	6.5 ± 0.16	0	12 ± 0.5	-	20 ± 0.5

Repeated chromatography of the EtOH or MeOH extracts from *T. letestui*, *L. edulis* and *Agaricus* sp. aff. *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.



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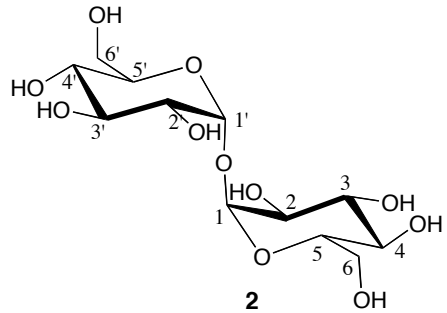

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5α,8α-Epidioxy-ergost-6,22-dien-3β-ol (5,8-peroxyergosterol) (**1**) exhibited the following properties: White crystals; m.p. 170 - 171°C, m.p. 178 – 179 °C (Kocor and Szalowska 1972); yield, 7 mg; R_f = 0.5 (20% EtOAc/hexane); anisaldehyde – blue; EI-MS, *m/z* (% rel. int.) 400 ([M – C_2H_4]$^+$, 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 $C_{28}H_{44}O_3Na$ = 451.31827; ^{1}H NMR, δ 0.80 (3H, *s*, H-18), 0.81 (3H, *s*, H-19), 0.90 (3H, *d*, *J* = 7 Hz, H-27), 0.92 (3H, *d*, *J* = 7 Hz, H-26), 0.99 (3H, *d*, *J* = 7 Hz, H-28), 1.01 (3H, *d*, *J* = 7 Hz, H-21), 1.22 (1H, *m*, H-17), 1.35 and 1.75 (2H, both *m*, H-16), 1.44 (1H, *m*, H-25), 1.50 (1H, *m*, H-14), 1.5 and 1.82 (2H, each *m*, H-15), 1.56 (1H, *m*, H-9), 1.58 (2H, *m*, H-11), 1.82 (2H, *m*, H-1), 1.84 (1H, *m*, H-20), 1.90 and 2.08 (2H, each *m*, H-4), 1.94 (2H, *m*, H-12), 2.06 (1H, *m*, H-24), 2.14 (2H, *m*, H-2), 3.97 (1H, *m*, H-3), 5.15 (1H, *dd*, *J* = 15.3, 8 Hz, H-23), 5.24 (1H, *dd*, *J* = 15.4, 8.2 Hz, H-22), 6.23 (1H, *d*, *J* = 8.6 Hz, H-6) and 6.49 (1H, *d*, *J* = 8.4 Hz, H-7); and ^{13}C NMR, δ 135.26 (C-6), 135.06 (C-23), 132.16 (C-22), 130.62 (C-7), 82.12 (C-5), 79.39 (C-8), 66.47 (C-3), 56.5 (C-17), 51.69 (C-9), 51.08 (C-14), 44.59 (C-10), 42.90 (C-20), 42.81 (C-13), 39.80 (C-24), 39.38 (C-12), 36.97 (C-4), 34.74 (C-2), 33.12 (C-25), 30.67 (C-15), 30.19 (C-1), 28.73 (C-21), 28.73 (C-16), 20.97 (C-28), 20.71 (C-11), 19.73 (C-19), 18.27 (C-27), 17.65 (C-26) and 12,97 (C-18).

α-D-Glucopyranosyl-(1→1)-α-D-glucopyranoside (α,α1,1′-trehalose, **2**) had the following properties: White crystals; mp. 120 – 123 °C trehalose dihydrate m.p. 97 – 99 °C (Matsuura *et al*. 2002); yield, 0.5 g; $[\alpha]_D^{29.1}$ = +197.29^O (c, 0.3, MeOH), $[\alpha]_D^{23}$ = +189.5^O (c, 0.20, MeOH) (Matsuura et al., 2002); anisaldehyde – no reaction; IR, ν_{max} (KBr) 3501, 2879-2991, 1457, 1398, 1354, 1332, 1311, 1240, 1211, 1149, 1128, 1098, 1084, 1061, 1030 and 1015 cm^{-1}; EI-MS, *m/z* (% rel. int.) 325 ([M-OH]$^+$, <10), 235 (10), 163 (30), 145 (20), 133 (15), 116 (7), 103 (100) and 74 (72): ESI-FT-ICR-MS (positive ion mode), *m/z* 365.10566 ([M + Na]$^+$), calc. for $C_{12}H_{22}O_{11}Na$ = 365.1054326; ^{1}H NMR, δ 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-6β′ and H-6β), 3.84 (2H, *m*, H-5 and H-5′), 3.87 (2H, *m,* H-3 and H-3′), 3.88 (*m*, H-6α and H-6α′), 5.19 (2H, *d, J* = 3.9 Hz, H-1 and H-1′) and ^{13}C NMR, δ 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 **1** 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 δ 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, Bok *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.

Termitomyces letestui polar fractions yielded α,α1,1′-trehalose (**2**) whose structure was deduced from ^{1}H- and ^{13}C-NMR spectral data (Breitmaeir and Voelter 1987, Duddeck *et al.* 1998), $[\alpha]_D$ and m.p. values (Matsuura *et al.* 2002) and positive ion ESI-FT-ICR MS that showed the molecular formula $C_{12}H_{22}O_{11}$ 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 *m/z* 325 resulting from cleavage of a hydroxyl unit from the molecular ion, and another peak at 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}C NMR spectrum indicated that the isolated compound was a symmetrical disaccharide.

α,α1,1′-Trehalose which is hereby being reported for the first time in a *Termitomyces* species was previously shown to possess α-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. letestui* for individuals susceptible to diabetes.

GC/MS analysis of the combined less polar fractions from *T. letestui* 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. letestui* 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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