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Chemical Constituents of *Scleroderma aurantium* I: A New Triterpene, 3, 25-Dihydroxy-22-acetoxyl-lanosta-8, 23-diene

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Abstract

A new lanostane type triterpene, 3,25-dihydroxy-22-acetoxyl-lanosta-8,23-diene was isolated from the fruit body of *Scleroderma aurantium*. The MeOH soluble-extract showed inhibitory activity on α-glucosidase enzyme (IC_{50} = 115 ppm) and antioxidant DPPH free radical scavenger activity (IC_{50} = 300 ppm). The isolate showed a weak inhibitory activity on α-glucosidase enzyme (IC_{50} = 320 ppm) and a week free radical scavenger (IC_{50} = 180 ppm).

Key words: *Scleroderma aurantium*, acetylated lanostane type triterpene, α-glucosidase inhibitor, DPPH free radical scavenger

Introduction

Scleroderma aurantium, vulgare, called Jamur So (in Indonesia/Java) is a yellow earth ball fungus belonging to the family of Sclerodermataceae Corda (Haksworth, *et al.*, 1995). The distribution of this mushroom is widespread, Europe, Asia and North America. In forest, the mushroom *S. aurantium* grows around *Gnetum gnemon* (Gnetaceae) plant, especially in the old plant. Sterols, glucose, fructose, maltose and indoles were reported as the chemical constituents of this mushroom (Nikoronov *et al.*, 1967). In ethanol extract, several free amino acid, alanine, asparagine, phenylalanine, α-aminobutyric acid, aspartic acid, glutamic acid, leucine, lysine, serine, tyrosine, and valine had been detected and identified using two dimensional paper chromatography (Grzybowska, 1967). The methanol extract contained indole-3-carboxylic acid, triptamine, and an aldehydic growth regulator (Ballester and Souza, 1972). Extraction of its peridium yielded a dihydroxy tetracyclic triterpene, 23φ-hydroxy lanosterol (Entwistle and Pratt, 1968), palmitic acid, linoleic acid, an ester of steroidal diol, ergosterol, ergosterol peroxide, 9(11)-dehydroergosterol peroxide, lanosta-8, 23- diene-3β, 25-diol, lanosta-8, 24- diene-3β, 23-diol, mannitol and 22-dihydroergosterol (Endo *et al.*, 1975; Vrkoc *et al.*, 1976).

Recently a number of steroids and triterpenes were isolated from various mushrooms. Most of the isolates were ergosterols and lanosterols. Some of the isolates showed antioxidant and anti-tumor activity (Shin *et al.*, 2000a; 2000b; 2001; Gerber *et al.*, 2000; Jones and Janardhanan, 2000). In our search for chemopreventive agents from Indonesian mushrooms, the methanol extract of the fruit body of *Scleroderma aurantium* showed an activity on free radical scavenger assay using a stable free radicals of α,α-diphenyl-β-picryl hidrazyl (DPPH). Furthermore, in searching for natural products anti-diabetic substances, the extracts were evaluated for their inhibition to α-glucosidase enzyme, in vitro. In this communication, we describe the isolation of a new active triterpene from the fruit body of *Scleroderma aurantium*.

Materials and Methods

Materials

The mushroom was collected in PUSPIPTEK Forest Provincial Garden in October 2001, identified as *Scleroderma aurantium*, vulgare, by one of us (TB). It is a common earth ball, hard sessile, peridium dirty olive yellow, scaly-cracked, thick. Fruit body tuberous, 4-8 cm, peridium in section whitish to rose-pink; spore mass finally purple black, traversed by whitish threads, powdery; spores escape by irregular cracking of peridium. Old fruit bodies are usually bored at base by beetles (Lange *et al.*, 1978). The dried fruit body was deposited in our laboratory.

Extraction and Isolation

Dried fruit body of *S. aurantium* (35 g) was pulverized and extracted with 5x500 ml MeOH to give 3.35 g of MeOH extract (9.57%). The MeOH extract was subsequently passed through a silica gel column chromatography (100 g of Si.gel; E-Merck) with the solvent system each of 250 ml of n-hexane; n-hexane-ethyl acetate 9:1; 8:2; 7:3; 6:4; and 5:5 then followed by ethyl acetate; ethyl acetate-methanol 9:1;

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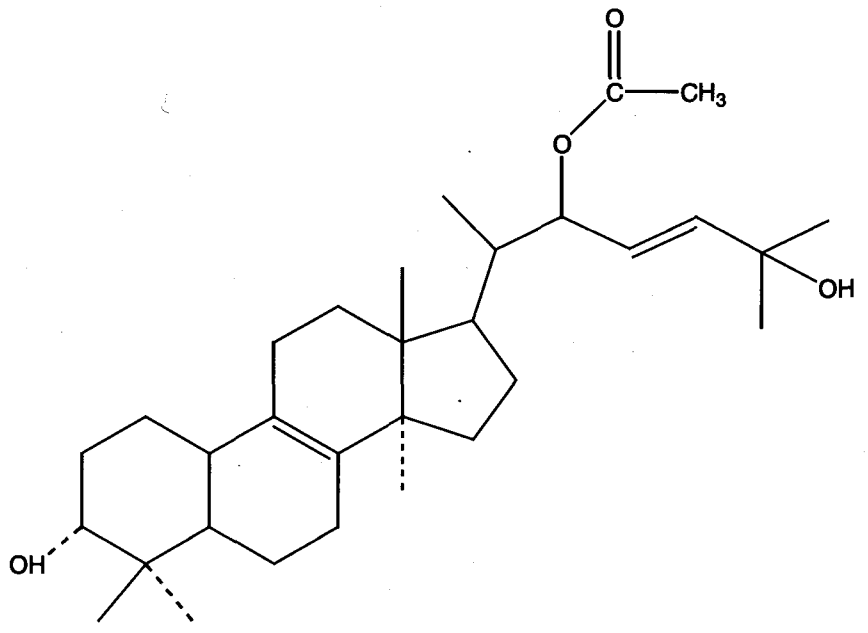
Table 1. DPPH Free Radical Scavenging Activity of MeOH-Extract and Fractions.

	DPPH Scavenging Activity (%)		
	200 ppm	40 ppm	8 ppm
MeOH Extract	29.79	10.23	0.42
Fraction 1 (F-1)	0.12	0.003	0.00
Fraction 2 (F-2)	0.026	0.023	0.006
Fraction 3 (F-3)	10.39	2.35	0.35
Fraction 4 (F-4)	88.89	55.02	12.17
Fraction 5 (F-5)	57.86	10.02	3.84
Fraction 6 (F-6)	83.91	20.28	10.68
Fraction 7 (F-7)	82.63	19.07	6.33
Fraction 8 (F-8)	45.55	16.94	-0.14
Fraction 9 (F-9)	10.18	5.76	3.06
Fraction 10	0.036	-0.06	0.005
BHA	92.31	86.9	32.03
BHT	85.69	53.59	19.71



Isolated compound: 3,25-dihydroxy-22-acetoxyl-lanosta-8,23-diene

7:3 and 5:5, consecutively, to give 10 fractions (F1-F10) which were combined based on their similarity of the thin layer chromatography pattern. These fractions were evaluated for their DPPH free radical scavenger activities, as shown in Table 1. Fraction F-5 (350 mg, IC_{50} = 180 ppm) was dissolved with 25 ml of n-hexane to yield n-soluble F-5 sub-fraction (200 mg). The n-hexane insoluble was dissolved in 25 ml acetone to yield F-5 acetone-soluble sub-fraction (75 mg). The acetone-insoluble was dissolved in MeOH and re-crystallized in MeOH-CH_2Cl_2 (1:1) to give white crystals (35 mg), as the isolate.

DPPH Free Radical Scavenging Activity

Assay for DPPH free radical scavenging activity: Reaction mixtures containing test samples (dissolved in DMSO) and 300 μM of α,α-diphenyl-β-picryl hidrazyl (DPPH) ethanol solution in 90-well microtiter plates were incubated at 37°C for 30 minutes and absorbance was measured at 515 nm (Yen and Chen, 1995). Percent inhibition by sample treatment was determined by comparison with DMSO-treated control group. The positive controls used were solutions of BHA, BHT and ascorbic acid. IC_{50} values denote the concentration of sample which is required to scavenge 50% DPPH free radicals. Butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) were used as the positive controls of which IC_{50} were 30 ppm and 50 ppm, respectively.

α-Glucosidase Inhibitory Activity

The inhibition of α-glucosidase evaluation was performed using a modified published method (Prasanth *et al.*, 2001). One mg of α-glucosidase (Saccharomyces sp., Oriental Yeast Co., Ltd.) was dissolved in 100 ml of phosphate buffer (pH 7.0) containing 200 mg of bovine serum albumin (Waco Pure Chemical Industries, Ltd.). This enzyme solution was diluted with the same buffer to 1/50 just before assay. The reaction mixture contained 500 μl of 20 nM p-nitrophenyl α-D-glucopyranoside (Waco Pure Chemical Industries, Ltd.), 990 μl of 100 mM phosphate buffer (pH 7.0) and 10 μl of test sample solution or DMSO. After the reaction mixtures were pre-incubated at 37°C for 5 minutes, the reaction was started by addition of 500 μl of the enzyme solution and incubated for exactly 15 minutes. After the incubation, this reaction was stopped by addition of 2000 μl of 200 mM Na_2CO_3 solution, and then the amount of released p-nitrophenol was measured the absorbance at 400 nm using a Hitachi U-2000 spectrometer. The inhibitory activity (%) was estimated as follows: [(C-S)/C]x100. "S" is the absorbance of a sample, and "C" is the absorbance of DMSO without the sample. Solution of 1 % quercetin was used as a positive control.

Analysis of Isolated Compound

Melting points were measured on a Buchi B-540 apparatus and uncorrected. UV and IR spectra were measured on a Hitachi U-2000 spectrometer in MeOH and on a Perkin Elmer FT-IR spectrometer in KBr, respectively. ^{1}H- and ^{13}C-NMR spectra were measured on a Brucker AMX-500 (^{1}H:500 MHz; ^{13}C:125 MHz) using deuterated chloroform ($CDCl_3$) as solvent, and tetramethylsilane (TMS) as an internal standard. One-dimensional (1D) NMR was performed with ^{1}H-, ^{13}C- (broad band decoupled spectra); and DEPT experiments. Two-dimensional (2D)-NMR was performed with ^{1}H-^{1}H, ^{1}H-^{13}C COSY, HMQC, HMBC and NOESY.

EIMS and HR-EIMS, CIMS (iso-butane for a gas) were measured on a Hitachi M-4100 instrument. TLC was performed using Silica gel 60 F_{254}, 0.25 mm (Merck), with detection provided by UV light (254 nm) and by spraying 10% H_2SO_4 solution, followed by heating, or 5% $FeCl_3$ reagent. Gravity column chromatography was performed using silica gel for column chromatography (Merck).

Results

Yield of Isolated Compound

The yields of the isolated compound, was 35 mg, (0.1 % w/w of dried mushroom).

Characteristics of Isolated Compound

The isolated compound was a white powder with the following characteristics: mp 196-198° C; Ir (KBr) ν 3400 (OH), 1720 (C=O), 1650 and 1560 (double bonds), 1050 (C-O-); R_f value on TLC 0.57 (solvent system: MeOH:CH_2Cl_2 1: 9); 0.34 (solvent system: n-hexane:EtOAc:MeOH 6:2:1); giving bluish purple color by 20% H_2SO_4 in EtOH spray reagent. HREI-MS: m/z 500.3838 [M^+] fits for the Molecular Formula of $C_{32}H_{52}O_4$; m/z 482.3743 ($C_{32}H_{50}O_3$ [M^+-H_2O]); m/z 422.3607 ($C_{30}H_{46}O$ [M^+-H_2O and CH_3COOH]; m/z 407 [M^+-H_2O-CH_3COOH-CH_3]; m/z 43 (base peak) for acetyl group. ^{1}H-NMR: δ 0.71 (3H, s, H-18), 0.83 (3H, s, H-29), 0.87 (3H, s, H-30), 0.94 (3H, d, J=7Hz, H-21), 0.97 (3H, s, H-28), 0.99 (3H, s, H-19), 1.21 (1H, m, H-5), 1.32 (3H, s, H-26), 1.33 (3H, s, H-27), 1.45 – 2.08 (m), 2.05 (3H, s, $\underline{CH_3}$ acetyl), 3.43 (1H, bs, H-3), 5.31(1H, dd, J=3.5; 8, H-22), 5.63 (1H, dd, J= 8, 16, H-23), 5.86 (1H, d, J=16, H-24); ^{13}C-NMR: δ 25.7 (C-1); 27.2 (C-2); 76.0 (C-3); 37.6 (C-4); 40.4 (C-5); 18.2 (C-6); 26.1(C-7); 134.8 (C-8); 133.8 (C-9); 36.9 (C-10); 20.9 (C-11); 29.7 (C-12); 44.6 (C-13); 49.5 (C-14); 30.9 (C-15); 30.8 (C-16); 47.1 (C-17); 22.2 (C-18); 19.0 (C-19); 40.4 (C-20); 13.2 (C-21); 76.8 (C-22); 120.8 (C-23); 142.1 (C-24); 70.7 (C-25); 30.1 (C-26); 29.9 (C-27); 28.0 (C-28); 15.8 (C-29); 24.1 (C-30); 21.5 (CH_3 Acetyl); 170.2 (C=O Acetyl).

Structural Determination

The isolate was determined to be a lanostane type triterpene by comparison of its NMR spectral data

with those of 3β,22,25-trihydroxy-lanosta-8-ene, isolated from *Inonotus obliquus* (Shin *et al.*, 2000 a;b). The compound had a hydroxyl group at a quarternary carbon at δ 7 ppm of C-25. The compound has two double bonds. First of the double bonds was at C-8 suggested by two ^{13}C-NMR resonance peaks at δ 134.8 and 133.8 ppm. In contrast with the first double bond of which carbons were quarternary, the second double bond is protonated. In HMBC-spectra, the ^{1}H-NMR resonances of the latter double bond has correlation with that of C-25, suggesting that these protons are of the doubule bond at C-23 and C-24. The HMQC spectra support that the olefine protons at δ 5.63 and 5.86 in ^{1}H-NMR have correlation with the signals at δ 120.8 and δ 147.1 in ^{13}C-NMR, respectively. The compound has an acetoxyl group attached to C-22 based on several reasons. In HMBC spectra, the ester carbonyl signal at δ 170.2 in ^{13}C-NMR has a crossed peak at δ 5.31 in ^{1}H-NMR. In HMQC spectra, this proton has a cross peak at δ 76.8 ppm in ^{13}C-NMR corresponding to C-22. In HMBC spectra, it has crossed peaks at δ 5.63 and δ 5.86 of the olefinic proton signals. The chemical shift of the methine proton of C-22 in ^{1}H-NMR and that of C-22 in and ^{13}C-NMR are shifted down in accordance with the suggested values (Knight, 1974; Cantrell *et al.*, 1996). The characteristic signal at δ 70.7 ppm in ^{13}C-NMR (Shin *et al.*, 2000a,b) suggests that one of the hydroxyl groups is attached at C-25. In HMBC spectra, the signal at δ 70.7 in ^{13}C-NMR has crossed peaks at δ 5.63 and δ 5.86, corrsponding to the protons on the double bond. The signal at δ 76.0 in ^{13}C-NMR is attributed to the second hydroxyl group attached to C-3. The configuration of the hydroxyl group at C-3 is considered to be in α position because the chemical shift of the signal in ^{13}C-NMR was 76.0 instead of 80.0 for β configuration (Shin *et al.*, 2000 a, b; 2001). This configuration was supported by a very small value of coupling constant of H-3 at δ 3.43 (bs) in ^{1}H-NMR. The configurations of hydroxyl group at C-25 (δ 70.7 in ^{13}C-NMR) and acetoxyl group at C-22 (δ 76.8 in ^{13}C-NMR; δ 5.32, dd, J = 8; 4 Hz in ^{1}H-NMR) were β configurations, in accordance with those of 3β,22,25-trihydroxy-lanosta-8-ene, a tri- terpene isolated from *Inonotus obliquus* (Shin *et al.*, 2000b). These configurations were also supported by the ^{1}H-^{1}H-NOESY spectrum. The proton at C-3 (δ 3.43) has correlation with methyl proton of C-28 (δ 0.97) and methyl proton of C-29 (δ 0.99).

Antioxidant Activity:

When the MeOH extract was dissolved in n-hexane, the n-hexane-soluble extract did not show any antioxidant activity. Table 1 show the antioxidant DPPH free radical scavenger activities of the MeOH extract and fractions. Among the fractions, F-4, F-6, F-9 and f-5 showed antioxidant activities (IC_{50} < 200 ppm). The new compound was firstly isolated from the F-5 by solvent fractionation and recrystallization. The IC_{50} value of the isolate was 180 ppm being same as that of the F-5. It indicated that the compound was the main antioxidant active constituent in the fraction.

Reports on the chemical constituents such as lanosterols and ergosterols suggest that this mushroom has potency as anticancer chemo-preventive agent. Further works on the F-4, F-6, and F-9 will be performed to identify the other antioxidant compounds.

α-Glucosidase Inhibitory Activity:

Inhibitors of glycosidase have many therapeutic potentials, including the treatment of cancer, diabetes and AIDS. If a compound showed an inhibitory activity to α-glucosidase enzyme, it has a potency as an antidiabetic or antiobesity drug candidate. In the course of the characterization studies on anti-obesitic and antidiabtogenic principles in mushrooms, we found that the MeOH extract of S. aurantium showed inhibitory activity on α-glucosidase enzyme (IC_{50} = 115 ppm). The isolated compound (IC_{50} = 320 ppm), however, showed less active compare to that of the MeOH extract, indicating that there were other components which are more active than the isolate. Further work will be performed to identify the more potent α-glucosidase inhibitors.

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