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Cytotoxic Chemical Constituents from the Mushroom of *Pholiota adiposa*

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Abstract The compounds, 1-Linoleic-2-olein (**1**), stigmasterol (**2**), 1,4-glucopyranosyl-1',4'-glucopyranosyl-1",4"-glucopyranoside (**3**), 2',3'-diphosphoryl-1'-propanoxy-β-D-glucopyranoside (**4**), 1-Linoleic-3-olein (**5**), 1-(N,N,N-trimethyl ethyl amino phosphoryl)-2,3-dilinolein ion (**6**) and glyceryl phosphate (**7**) were isolated and identified from the Mushroom of *Pholiota adiposa* for the first time by column chromatography, TLC, UV, IR, ^{1}H and ^{13}C NMR, EI-MS, and FAB-MS. The compounds **1** and **2** were found to have weak cytotoxicity against P388 murine leukemia cells. However, the compounds **6** and **7** did not show any cytotoxicity.

Keywords: *Pholiota adiposa*, mushroom glutinous, mushrooms compounds, cytotoxic activity.

Introduction

For a long time mushrooms have been valued as flavorful foods with medicinal properties. They are widely sold as nutritional supplements and touted as beneficial for health. A number of bioactive molecules, including terpenoids, steroids, phenols, nucleotides and their derivatives, glycoprotein and polysaccharides, have been identified in numerous mushroom species (1, 2). Edible mushrooms owe their taste primarily to the presence of small quantities of several water-soluble substances, including 5'-nucleotides, and soluble carbohydrates (3, 4). The edible mushroom *P. adiposa* is cultivated in several regions of Asia, Europe and North America (5-6). However, little information has been available about the functional components of *P. adiposa* and much more research is required to identify the bioactive constituents. Recently, morphological features and dietary functional components in fruit bodies of *P. adiposa* have been reported (7, 8). The objective of this study was to isolate and identify the functional components of *P. adiposa*. All the compounds herein are reported for the first time and some compounds were also studied cytotoxicity.

Materials and Methods

General experimental procedures Column chromatography (CC) was performed in silica gel (70-230 mesh, Merck) and Lichroprep RP-18 (ODS silica gel, Merck). Preparative thin-layer chromatography (TLC) was performed on glass plates (20 mm×20 mm×0.2 mm) coated with silica gel (60 GF254, Merck). The compounds were visualized by dipping the plates into 1% vanillin-sulfuric acid-ethanol solution followed by heating at 100-110°C for 5-10 min. Optical rotations were measured on an AA-10 model polarimeter. UV spectra were recorded with spectrophotometer TU-1800$_{PC}$. IR spectra were run on a Thermo Mattson 60 AR spectrophotometer. Both ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were conducted on a Bruker Avance DRX-500 MHz spectrometer with tetramethylsilane (TMS) as internal standard. EI-Mass spectra were recorded on a JEOL JMS-SX102A and FAB-MS on a JEOL-JMS-AX 505 WA spectrometer.

Isolation of compounds from *Pholiota adiposa* (Mushroom) The *Pholiota adiposa* samples were collected from the Rural Development Administrate, Suwon, Korea (8). The mushroom was freeze dried and ground in powder form to give 302 g solid, which was extracted with [hexane-EtOAc-MeOH; 1:1:1] (1.5 L×3) at room temperature. After removal of solvent in vacuo, the extract (11.5 g) was chromatographed over silica gel 60 using n-hexane, EtOAc, n-hexane-EtOAc and EtOAc-MeOH mixtures of increasing polarity and finally methanol gave the following fractions: frs. 1-4 in hexane, frs. 5-8 in hexane–EtOAc (9:1), frs. 9-12 in hexane-EtOAc (8:2), frs. 13-16 in hexane-EtOAc (1:1), frs. 17-20 in hexane-EtOAc (1:3), frs. 21-24 in EtOAc, frs. 25-28 in EtOAc-MeOH (9:1), frs. 29 - 34 in EtOAc-MeOH (1:1), frs. 35-39 in MeOH.

Fr. 3 was further purified by CC over Si gel with methylene chloride and methanol to yield a pure compound after preparative TLC and was identified as 1-Linoleic-2-olein (**1**, 20 mg; R_f 0.73; Hex:EtOAc; 8:2) on the silica gel plates developed by a solvent. Fr 7 was crystallized and after purification through CC to yield and was identified as stigmasterol (**2**, 50 mg; R_f 0.38; $CHCl_3$: MeOH; 9.5:0.5), confirmed by comparison to an authentic sample from sigma. Fr 29 was further purified by CC over Lichroprep RP-18 (ODS silica gel) with water and methanol to afford two pure compounds: 1,4-glucopyranosyl-1',4'-glucopyranosyl-1",4"-glucopyranoside (**3**, 50 mg) and 2',3'-diphosphoryl-1'-propanoxy-β-D-glucosides (**4**, 20 mg). Fr 34 was further purified by CC over Lichroprep RP-18 (ODS silica gel) with water and methanol to afford one pure compound: 1-linoleic-3-olein (**5**, 30 mg). Fr 37 was further purified by CC over Lichroprep RP-18 (ODS silica gel) with water and methanol to afford two pure compounds: 1-N,N,N-trimethyl ethyl amino phosphoryl)-2,3-dilinolein ion (**6**, 100 mg) and glyceryl phosphate (**7**, 20 mg). NMR results indicated that the several other

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Table 1. ^{1}H NMR and ^{13} C-NMR of stigmasterol (2) ($CDCl_3$, ^{1}H NMR-500 MHz, ^{13}C NMR-125 MHz)

position	^{13}C	^{1}H
1	37.27	1.04, 1H, m; 1.87, 1H, m
2	32.23	1.56, 1H, m; 1.89, 1H, m
3	71.70	3.62, 1H , m
4	43.55	2.05 & 2.28, 1H, dd each
5	140.80	----------
6	121.69	5.22, 1H, m ,
7	31.94	2.45, 2H, m
8	31.94	1.48, 1H, dd
9	50.20	1.25,1H, m
10	36.56	----------
11	21.11	1.04, 1H, m; 1.87, 1H, m
12	39.74	1.04, 1H, m; 1.87, 1H, m
13	42.35	----------
14	56.91	1.30, 1H, m
15	24.39	1.48, 2H, m
16	28.96	1.89, 1H, m; 1.30, 1H, m
17	56.06	1.14, 1H, m
18	12.28	0.62, 3H, s
19	19.42	0.92, 3H, s
20	40.54	1.69, 1H, m
21	21.11	0.92, 3H, d
22	138.37	5.56,1H, m
23	129.32	5.37, 1H, m
24	51.29	1.34, 1H, m
25	31.94	1.77, 1H, m
26	21.26	0.80, 3H, d
27	19.02	0.90, 3H, d
28	25.44	1.25, 1H, m; 1.38, 1H, m
29	12.27	0.88, 3H, d

fractions contained only sugar molecules.

1-Linoleic-2-olein (1). EI-MS : m/z 618 $[M]^+$; (calc for $C_{39}H_{70}O_5$); FAB-MS: m/z 619 $[M+H]^+$.

Stigmasterol (2). m.p. 165-167°C; IR bands (KBr) 3430, 2956, 1653, 1459, 1373, 1035; EI-MS m/z 412 $[M]^+$ (calc for $C_{29}H_{48}O$) (0.8), 396 (100), 394 $[M^+-H_2O]^+$ (4.5), 378 (19), 363 (98), 337 (43), 271 (27), 253 (57), 239 (8.9), 227 (9), 211 (22), 199 (17), 171 (16.7), 157 (30), 143 (26.3), 131 (11.6),119 (15), 107 (15.5), 95 (13.6), 81 (26.5), 69 (43.4). ^{1}H and ^{13}C-NMR (see Table 1).

Table 2. Growth inhibition by 50% (IC_{50}) values of the known compounds against P388 murine leukemia cells

Compound	IC_{50} (μg/mL)
1-Linoleic-2-olein	41[a1)]
Stigmasterol	50[a]
1-(N,N,N-trimethyl ethyl amino phosphoryl)-2,3-dilinolein ion	100[b]
Glyceryl phosphate	100[b]

[1)]Statistical significance was analyzed by LSD (Least Significant Difference) ($P<0.05$).

1,4-glucopyranosyl-1',4'-glucopyranosyl-1",4"-glucopyranoside (3). EI-MS: m/z 504 (calc for $C_{18}H_{32}O_{16}$); FAB-MS: m/z $[M+H]^+$ ion peak at m/z 505.

2',3'-diphosphoryl-1'-propanoxy-β-D-glucopyranoside (4) EI-MS: m/z 414 (calc for $C_9H_{20}O_{14}P_2$). FAB-MS m/z $[M+H]^+$ ion peak at m/z 415.

1-Linoleic-3-olein (5). EI-MS: m/z 618 $[M]^+$; (calc for $C_{39}H_{70}O_5$); FAB-MS: m/z 619$[M+H]^+$.

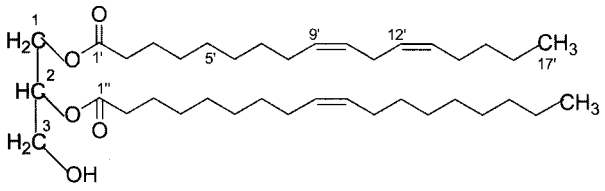
1-(N,N,N-trimethyl ethyl amino phosphoryl)-2,3-dilinolein ion (6). EI-MS: m/z 782 $[M]^+$; (calc for $C_{44}H_{81}O_8$ PN); 804 $[M+Na-H]^+$; FAB-MS: m/z 783 $[M+H]^+$; ^{1}H-NMR spectrum : δ 3.68 (H-5), 3.91 (H-3), 4.10 (H-3), 4.26 (H-4), 4.38 (H-1), 5.17 (H-2), 3.21 (H-6), 2.76 H-11', 11"), 2.28 (H-2',2"), 2.03 (H-8', 8", 14', 14"), 1.56 (H-3', 3"), 1.29 (H- 4'~H-7', H- 4"~H-7", H-15', H-16', H-15", H-16"), 0.88 (H-18', 18"). ^{13}C NMR: δ 63.1 (C-1), 70.6 (C-2), 63.7 (C-3), 59.4 (C-4), 66.4 (C-5), 54.9 (C-6), 34.4, 34.2 (C-2', 2"), 27.4, 27.3 (C-8', 8", 14', 14"), 14.28, 14.24 (C-18', 18")

Bioassay for cytotoxic activity Cytotoxic assays were performed by using the MTT assay method (9). The murine P388 leukemia cells were cultured in RPMI 1640 medium (Nissui) supplemented with 5% heat-inactivated, fetal bovine serum (FBS) and Kanamycin (5.3 mL/L) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The 100 μL of cell suspension was added to each well (3 X 10^3 cells/well) of a 96-microwell plate (Iwaki, flat bottom, treated polystyrene) which was incubated for 24 h. Test compounds were dissolved in DMSO at various concentrations (100, 30, 10, 3, 1, 0.3, 0.1 μg/mL) and 10 μL of the test solution or DMSO (control) was added to each well. The plate was kept in an incubator for 48 h. After termination of cell culture by adding 20 μL MTT (5 % in PBS) to each well, the plate was further incubated for 4 h. To each well was added 100 μL of 10% SDS-0.01N HCl. The plate was read on a microplate reader (MPR A4i, Tosoh) at 550 nm. A dose-response curve was plotted for each compound, and the concentrations inhibiting cell growth by 50% (IC_{50}) were recorded.

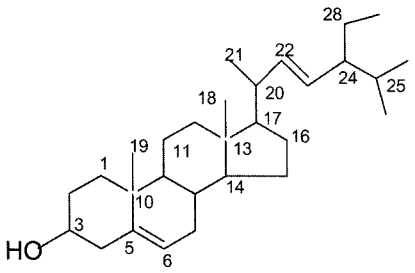
Results and Discussion

Identification of the chemical constituents The extracts of mushroom (*P. adiposa*) were separated as described in the experimental section to yield 7 known compounds, all of which were isolated for first time from *P. adiposa*. Compound **1** was obtained as a yellow liquid which exhibited a peak at m/z 618 in EI-MS (calc for $C_{39}H_{70}O_5$). FAB-MS of **1** gave an $[M+H]^+$ ion peak at m/z 619 and a molecular ion peak of 618 was also suggested. The IR spectrum of **1** gave bands at 3390, 1710, 1080, and 897 cm^{-1}. Analysis of **1** by IR, ^{1}H and ^{13}C NMR supported the structure of 1-Linoleic-2-olein.

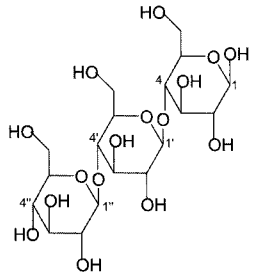
Compound **2** was obtained as colorless needle shaped crystals with a peak at 412 $[M^+]$ (calc for $C_{29}H_{48}O$) which suggested a molecular ion peak. The IR spectrum gave bands at 3430, 1653, 1459, 1373, and 1035 cm^{-1} with the first band being characteristic for hydroxyl group. Analysis of **2** by IR, ^{1}H and ^{13}C NMR supported the structure of



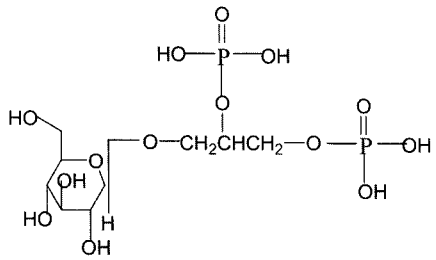
1-Linoleic-2-olein (1)



Stigmasterol (2)



1,4-glucopyranosyl-1',4'-glucopyranosyl-1'',4''-glucopyranoside.(3)



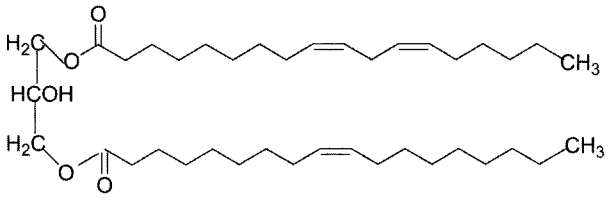
2',3'-Diphosphoryl-1'-propanoxy-β-D-glucopyranoside (4)

stigmasterol.

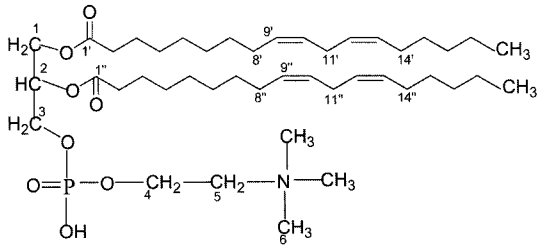
Compound **3** was obtained as a yellow liquid which exhibited a peak at m/z 504 in EI-MS (calc for $C_{18}H_{32}O_{16}$). FABMS of **3** gave an $[M+H]^+$ ion peak at m/z 505 and a molecular ion peak of 504 was also suggested. Analysis of **3** by IR, ^1H and ^{13}C NMR supported the structure of 1,4-glucopyranosyl-1',4'-glucopyranosyl-1'',4''-glucopyranoside.

Compound **4** was obtained as a white gummy substance which exhibited a peak at m/z 414 in EI-MS (calc for $C_9H_{20}O_{14}P_2$). FAB-MS of **4** gave an $[M+H]^+$ ion peak at m/z 415 and a molecular ion peak of 415 was also suggested. Analysis of **4** by IR, ^1H and ^{13}C NMR supported the structure of 2',3'-diphosphoryl-1'-propanoxy-β-D-glucopyranoside.

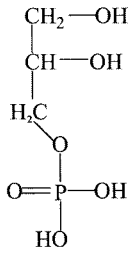
Compound **5** was obtained as a yellow liquid which



1-Linoleic-3-olein (5)



1-(N,N,N-trimethyl ethyl amino phosphoryl)-2,3-dilinolein ion (6)



Glyceryl phosphate (7)

exhibited a peak at m/z 618 in EI-MS (calc for $C_{39}H_{70}O_5$). FAB-MS of **5** gave an $[M+H]^+$ ion peak at m/z 619 and a molecular ion peak of 618 was also suggested. Analysis of **5** by IR, ^1H and ^{13}C NMR supported the structure of 1-Linoleo-3-olein.

Compound **6** was obtained as a yellow gummy mass which exhibited a peak at m/z 782 in EI-MS (calc for $C_{44}H_{81}O_8PN$). FAB-MS of **6** gave an $[M+H]^+$ ion peak at m/z 783 and a molecular ion peak of 782 was also suggested. Analysis of **6** by IR, ^1H and ^{13}C NMR supported the structure of **1**-(N,N,N-trimethyl ethyl amino phosphoryl)-2,3-dilinolein ion.

Compound **7** was obtained as a crystalline solid which exhibited a peak at m/z 172 in EI-MS (calc for $C_3H_9O_6P$). FAB-MS of **7** gave an $[M+H]^+$ ion peak at m/z 173 and a molecular ion peak of 172 was also suggested. ^1H and ^{13}C NMR analysis of **7** supported the structure of glyceryl phosphate.

Cytotoxic activity The compounds 1-Linoleic-2-olein (**1**) and stigmasterol (**2**) showed weak cytotoxicity against P388 murine leukemia cells and other 1-(N,N,N-trimethyl ethyl amino phosphoryl)-2,3-dilinolein ion (**6**) and glyceryl phosphate (**7**) did not show any cytotoxicity. From other researches, additionally, it is known that stigmasterol has antiinflammatory (10), hypocholesterolemic (11), and cytostatic activity (12).

Acknowledgments

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