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Chlorinated Hydroquinone Derivatives of Fruiting Body of *Russula subnigricans*[*1]

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ABSTRACT

The 95% aqueous EtOH extract was obtanied from the fruiting body of *Russula subnigricans*. Repeated silica gel column chromatography and preparative TLC afforded one fatty acid and three chlorinated hydroquinone derivatives. They were identified as nonadecanoic acid **(1)**, 2,6-dichloro-4-methoxyphenol **(2)**, russuphelin A **(3)**, and russuphelin E **(4)** on the basis of several spectral data (MS, ^{1}H and ^{13}C-NMR, including HMBC).

Keywords : *Russula subnigricans*, peparative TLC, chlorinated hydroquinone, russuphelin A, russuphelin E

1. INTRODUCTION

Russula subnigricans is a basidiomycete mushroom which was found in Asia and was named by Japanese mycologist Tsuguo Hongo in 1955 and shares characteristics of the North American fungus *R. eccentrica*. Ingestion of the mushroom has in recent years led to a spate of mushroom poisonings in Japan and elsewhere. Initial symptoms include nausea and diarrhea, which can start within half an hour of eating the toxic mushrooms (Kim *et al*., 2009). *R. subnigricans* has ivory to brown cap, ivory stem and distant gills staining reddish brown gradually. Poisoning with this mushroom has been known to occur because of its similarity to *R. nigricans*, an edible mushroom (Imazeki and Hongo, 1989; Ohta *et al*., 1995). The potential toxicity of these mushrooms has been known in Japan since 1954, and chemical constituents have been previously isolated and identified several amino acids ((2*S*,3*R*)-(-)-3-hydroxybaikiain, (*S*)-(-)-baikiain, (*S*)-(-)-pipecolic acid) and sterol derivatives (ergosterol, ergosteryl peroxide, cerevisterol) (Kusano *et al*., 1987). Chlorinated hydroquinone derivatives, russuphelol, russuphelin A, B, C, D, E and F were identified as cytotoxic compounds (Takahashi *et al*., 1992, 1993; Ohta *et al*., 1995). Cycloprop-2-ene carboxylic acid is fairly well known to synthetic organic chemists but has never before been observed in a biological system (Kim *et al*., 2009).

Poisonous mushrooms have attracted the attention of many scientists because of the unique

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chemical structures and the remarkable biological properties of their toxic components. At the present, a variety of mushroom toxins have been characterized, and some of them have become useful for biomedical research. Poisonous mushrooms can serve for a resource of biomedical application. Therefore, this study was carried out to investigate the chemical constituents of fruiting body of *R. subnigricans*, which was separated by preparative TLC method. Their chemical structures were identified by spectroscopic methods including ^{1}H-, ^{13}C-NMR, HMBC, MALDI-TOF MS and EI-MS.

2. MATERIALS and METHODS

2.1. General Experimental

^{1}H- and ^{13}C-NMR spectra were measured with a Bruker DPX 400 (Germany) spectrometer in DMSO-d_6 and $CDCl_3$ operating at 400 MHz and 100 MHz, repectively. MALDI-TOF MS spectrum was measured with a Voyager-DE STR mass spectrometer. EI-MS was recorded on a Micromass Autospec M363 spectrometer. Medium pressure liquid chromatography (MPLC) was carried out by a Combiflash Retrieve (ISCO) apparatus with the columns containing Silica gel (230～400 mesh). Preparative TLC was preformed on Silica gel 60 F_{254} glass plates (1 mm, 20 × 20 cm) (Merck), developed with dichloromethane (DCM)-MeOH (3 : 1, v/v) and benzene-acetone (8 : 2, v/v), and visualized by UV light (254 and 365 nm).

2.2. Material

The fruiting body of *Russula subnigricans* was collected from the Research Forest of Kangwon National University in July, 2008. The species was identified by Professor Jong-Kyu Lee of the Department of Forest Resources Protection.

2.3. Extraction and Isolation

The 42.6 g of fruiting body of *R. subnigricans* were cut into small piece and extracted 3 times with 95% aqueous EtOH (1 ℓ) at room temperature. After filtration (Advantec No. 2), filtrates were combined and evaporated on a rotary evaporator under the reduced pressure at 40°C. The extracts of *R. subnigricans* (4 g) were chromatographed on a Silica gel column (40 g, 3 × 15 cm) using a gradient solvent system of hexane-EtOAc (3 : 1～1 : 1, v/v) to give 3 fractions (RS1～RS3). RS1 fraction was applied to a preparative TLC with DCM-MeOH (3 : 1, v/v) to afford compounds **1** (19 mg), **2** (13 mg) and **3** (25 mg). RS2 and RS3 fractions were reapplied to a preparative TLC with benzene-acetone (8 : 2, v/v) to afford compounds **3** (20 mg) and **4** (15 mg).

2.3.1. Nonadecanoic Acid (1)

EI-MS : Calculated for $C_{19}H_{38}O_2$ 298, Found *m/z* 298 $[M]^+$.

^{1}H-NMR (400 MHz, δ, $CDCl_3$) : 0.88 (3H, *t*, *J* = 6.5 and 7.0 Hz, H-19), 1.25～1.30 (30H, *br s*, H-4～H-18), 1.63 (2H, *m*, H-3), 2.35 (2H, *t*, *J* = 7.5 and 7.5 Hz, H-2).

^{13}C-NMR (100 MHz, δ, $CDCl_3$) : 14.13 (C-19), 22.71 (C-18), 24.69 (C-3), 29.08 (C-4), 29.38 (C-5), 29.45 (C-16), 29.61 (C-6), 29.71 (C-7～C-15), 31.95 (C-17), 34.05 (C-2), 180.05 (C-1).

2.3.2. 2,6-dichloro-4-methoxyphenol (2)

EI-MS : Calculated for $C_7H_6Cl_2O_2$ 192, Found *m/z* 298 $[M]^+$, 194 $[M+2]^+$, and 196 $[M+4]^+$.

^{1}H-NMR (400 MHz, δ, DMSO-d_6) : 3.54 (3H, *s*, 4-OCH_3), 6.63 (2H, *s*, H-3,5).

^{13}C-NMR (100 MHz, δ, DMSO-d_6) : 56.35 (4-OCH_3), 114.27 (C-3,5), 122.06 (C-2,6), 141.12 (C-1), 151.09 (C-4).

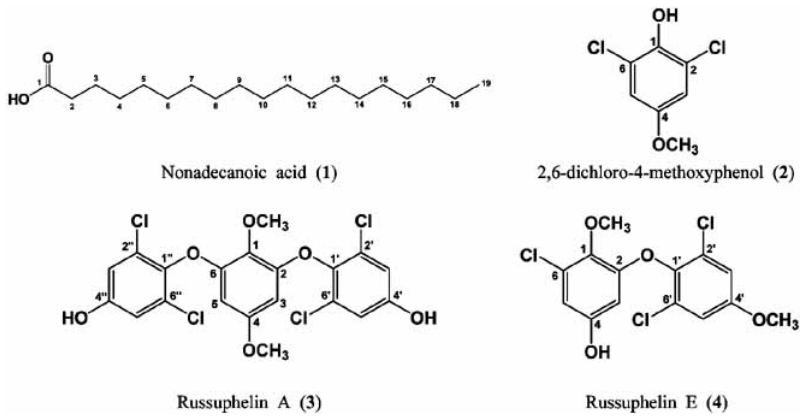

Fig. 1. The chemical structures of compounds **1-4**.

2.3.3. Russuphelin A (3)

MALDI-TOF MS : Calculated for $C_{20}H_{14}O_6Cl_4$ 490, Found *m/z* 490 $[M]^+$, 492 $[M+2]^+$, 494 $[M+4]^+$, 496 $[M+6]^+$, and 498 $[M+8]^+$.

^{1}H-NMR (400 MHz, δ, DMSO-d_6) : 3.42 (3H, *s*, 4-OCH_3), 3.89 (3H, *s*, 1-OCH_3), 5.57 (2H, *s*, H-3,5), 6.94 (4H, *s*, H-3′,5′,3″,5″).

^{13}C-NMR (100 MHz, δ, DMSO-d_6) : 55.63 (4-OCH_3), 60.94 (1-OCH_3), 93.56 (C-3,5), 116.76 (C-3′,5′,3″,5″), 128.60 (C-2′,6′,2″,6″), 130.91 (C-1), 136.61 (C-1′,1″), 151.97 (C-2,6), 155.60 (C-4′,4″), 158.30 (C-4).

2.3.4. Russuphelin E (4)

EI-MS : Calculated for $C_{14}H_{11}Cl_3O_4$ 347, Found *m/z* 347 $[M]^+$, 349 $[M+2]^+$, and 351 $[M+4]^+$.

^{1}H-NMR (400 MHz, δ, DMSO-d_6) : 3.64 (3H, *s*, 4′-OCH_3), 3.85 (3H, *s*, 1-OCH_3), 5.81 (1H, *d*, J = 2.8 Hz, H-5), 6.77 (1H, *d*, J = 2.8 Hz, H-3), 7.08 (2H, *s*, H-3′,5′).

^{13}C-NMR (100 MHz, δ, DMSO-d_6) : 55.70 (4′-OCH_3), 60.50 (1-OCH_3), 99.79 (C-3), 106.99 (C-5), 116.11 (C-3′,5′), 128.26 (C-2′,6′), 129.52 (C-6), 137.05 (C-1), 138.01 (C-1′), 151.08 (C-2), 155.43 (C-4′), 155.85 (C-4).

3. RESULTS and DISCUSSION

Compound **1** was obtained as a white amorphous powder, and R_f value was 0.55 (DCM-MeOH (3 : 1, v/v)). EI-MS spectrum gave a molecular ion of *m/z* 298 $[M]^+$ and suggesting a possible molecular formula of $C_{19}H_{38}O_2$. The ^{1}H-NMR spectrum of **1** indicated the presence of saturated fatty acid signals as several aliphatic methylene protons at δ 1.25～1.30 (H-4～H-13), δ 1.63 (H-3) and 2.35 (H-1), and one methyl protons at δ 0.88 (H-19). The ^{13}C-NMR spectrum of **1** indicated the presence of one carbonyl carbon at δ 180.05 (C-1), one methyl carbon at δ 14.13 (C-19) and several methyl carbons. The structure of **1** was identified as nonadecanoic acid based on the above consideration and a comparison with reported data (Al Dulayymi *et al*., 2005; Budimir *et al*., 2007). Compound **2** was obtained as a brownish amorphous powder, and R_f value was 0.70 (DCM-MeOH (3 : 1, v/v)). The molecular formula was deduced to be $C_7H_6Cl_2O_2$ on the basis of the peak at *m/z* 192 $[M]^+$, 194 $[M+2]^+$, and 196 $[M+4]^+$ in the EI-MS. When chlorine is present, the M+2 peak becomes very significant. If a compound contains two chlorine atoms, a quite

distinct M+4 peak, as well as an intense M+2 peak (Pavia *et al*., 2001). The ^{1}H-NMR spectrum of **2** showed a singlet signal at δ 6.63 due to a pair of aromatic protons, and one methoxyl protons at δ 3.54. The ^{13}C-NMR spectrum of **2** exhibited a methoxyl carbon at δ 56.35, two pairs of symmetric aromatic carbons at δ 114.27 (C-3,5) and δ 122.06 (C-2,6), and two oxygenated aromatic carbons at δ 141.12 (C-1) and δ 151.09 (C-4). According to the above data, compound **2** was identified as 2,6-dichloro-4-methoxyphenol (Takahashi *et al*., 1993; Haggblom *et al*., 1988; Knuutinen *et al*., 1988).

Compound **3** was obtained as a brownish amorphous powder, and R_f value was 0.60 (DCM-MeOH (3 : 1, v/v)) and 0.52 (benzene-acetone (8 : 2, v/v)). The molecular formula $C_{20}H_{14}O_6Cl_4$ was deduced from MALDI-TOF MS. The presence of four chlorine atoms was indicated by the molecular ion cluster at *m/z* 490 $[M]^+$, 492 $[M+2]^+$, 494 $[M+4]^+$, 496 $[M+6]^+$, and 498 $[M+8]^+$. (Takahashi *et al*., 1992). The ^{1}H-NMR spectrum of **3** displayed only four singlet signals assignable to two methoxyl protons (δ 3.42, δ 3.89) and six aromatic protons (δ 6.94 (4H, H-3′,5′,3″,5″), δ 5.57 (2H, H-3,5)). The ^{13}C-NMR spectrum of **3** revealed only ten carbon signals due to two methoxyl and eight aromatic carbons, accounting for half of the all carbons. In support of the above assignments, long-range H-C couplings (HMBC) were observed 1,4-OCH_3 and C-1,4, H-3,5 and C-1′,1″, and H-3′,5′,3″,5″ and C-1′,1″ which confirmed the proper structure linkage as shown in Fig. 1. According to the combination of spectroscopic data and literature (Takahashi *et al*., 1992, 1993; Ohta *et al*., 1995), compound **3** was elucidated as russuphelin A (2,6-bis(2,6-dichloro-4-hydroxyphenyloxy)-1,4-dimethoxybenzene). Compound **4** was obtained as a brownish amorphous powder, and R_f value was 0.65 (benzene-acetone (8 : 2, v/v)). The ^{1}H- and ^{13}C-NMR spectrum is similar to those for compound **3**, except for the absence of a presence of a chlorinated phenyl moiety. The ^{1}H-NMR spectrum of **4** showed two doublet signals at δ 5.81 (2.8 Hz, H-5), δ 6.77 (2.8 Hz, H-3) due to a *meta-coupling* of non-symmetric structure. The molecular formula $C_{14}H_{11}Cl_3O_4$ was supported by molecular ion peak at *m/z* 347 $[M]^+$, 349 $[M+2]^+$, and 351 $[M+4]^+$ from EI-MS. According to the above data, compound **4** was identified as russuphelin E (Takahashi *et al*., 1993).

The present study reported the isolation of one fatty acid (**1**) and three chlorinated hydroquinones (**2**,**3**,**4**) from the fruiting body of *R. subnigricans*. Nonadecanoic acid (**1**) has been known to be isolated from several sources such as fungi, marine sponges, plants and larvae (Yoo *et al*., 2002), and it was characterized for the first time from *R. subnigricans*. 2,6-dichloro-4-methoxyphenol (**2**) has been isolated from *Rhodococcus chlorophenolicus* (Haggblom *et al*., 1988), *Phanerochaete chrysosporium* (Reddy *et al*., 1998) and *R. subnigricans* (Takahashi *et al*., 1993). Russuphelin A (**3**) and russuphelin E (**4**) have only been found in *R. subnigricans* (Takahashi *et al*., 1993). 2,6-dichloro-4-methoxyphenol (**2**), russuphelin A (**3**) and russuphelin E (**4**), based on chlorinated hydroquinone unit, can be important chemotaxonomic markers of *R. subnigricans*, suggesting that they could be potential toxins.

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