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Lactarane sesquiterpenoids from *Lactarius subvellereus* and their cytotoxicity

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ABSTRACT

Subvellerolactones B (**1**), D (**2**), and E (**3**), structurally unusual lactarane sesquiterpenoids, were isolated from the fruiting bodies of *Lactarius subvellereus* together with four known lactarane sesquiterpenes (**4**–**7**). The chemical structures and stereochemistries of compounds **1**–**3** were determined on the basis of spectroscopic analyses, including 1D and 2D NMR experiments and a convenient Mosher ester procedure. Subvellerolactone B (**1**) exhibited cytotoxicity against the A549, SK-MEL-2, and HCT-15 cell lines with IC_{50} values of 26.5, 18.3, and 14.2 μM, respectively, and subvellerolactones D (**2**) and E (**3**) showed cytotoxicity against the A549 and HCT-15 cell lines (IC_{50} (**2**): 25.1 and 17.8 μM, and IC_{50} (**3**): 19.6 and 28.7 μM, respectively).

Mushrooms are a rich source of chemically interesting and biologically significant secondary metabolites.[1,2] Despite their potential for scientific investigation, few bioactive metabolites have been reported from mushrooms compared with higher plants. During the last three years, our research has focused on secondary metabolites from poisonous Korean mushrooms.[3–5] In our continuing search for structurally interesting and bioactive natural products from Korean mushrooms, we investigated the constituents of *Lactarius subvellereus* (Russulaceae), inedible mushroom that contains several lactarane sesquiterpenes.[6,7] Chemical investigation on the fruiting bodies of *L. subvellereus* resulted in the isolation of three new lactarane sesquiterpenoids, subvellerolactones B (**1**), D (**2**), and E (**3**), together with four known lactarane sesquiterpenes (**4–7**) (Fig. 1). In this Letter, we describe the isolation, structural elucidation of three new lactarane sesquiterpenoids (**1**–**3**), and cytotoxic activities of the isolates (**1**–**7**).

The fresh fruiting bodies of *L. subvellereus* were collected at Yongin, Gyeonggi-do, Korea, in November, 2005, and the mushroom was identified by one of the authors (S.J.S.). A voucher specimen (SKKU 2005-11c) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

The air-dried and powdered fruiting bodies of *L. subvellereus* (8 g) were extracted with 80% aqueous MeOH two times at room temperature. The extract was filtered, concentrated, and partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH, successively, yielding *n*-hexane (350 mg), $CHCl_3$ (100 mg), and *n*-BuOH-soluble fractions (210 mg). We investigated the $CHCl_3$ and *n*-BuOH-soluble fractions which showed considerable cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cell lines in screening procedures. The $CHCl_3$-soluble fraction (100 mg) was subjected to fractionation with column chromatography (Sephadex LH-20, 100% MeOH) and purified by reversed-phase HPLC using a Gilson 306 pump with a Shodex refractive index detector (Econosil RP-18 10μ column, 70% aqueous MeOH) to afford compounds **4** (5 mg), **5** (4 mg), **6** (5 mg), and **7** (4 mg). The *n*-BuOH-soluble fraction (210 mg) was also subjected to fractionation with column chromatography (Sephadex LH-20, 100% MeOH) and purified by reversed-phase HPLC (Econosil RP-18 10μ column, 60% aqueous MeOH) to give compounds **1** (6 mg), **2** (5 mg), and **3** (5 mg). The known lactarane sesquiterpenes were identified as 5-desoxylactarolide B (**4**),[8] furandiol (**5**),[8] lactarorufin A (**6**),[8] and lactarolide A (**7**),[8] by comparison of their spectroscopic data to previously reported values and, to the best of our knowledge, they were isolated for the first time from this mushroom.

Subvellerolactone B (**1**)[9] was obtained as a colorless oil. The molecular formula of **1** was deduced to be $C_{15}H_{22}O_5$ by positive mode HR-FABMS data at *m*/*z* 305.1357 $[M+Na]^+$ (calcd for $C_{15}H_{22}NaO_5$, 305.1365). The IR spectrum of **1** exhibited a typical lactonic absorption band at 1768 cm^{-1}, as well as absorption bands for hydroxyl groups (3387 cm^{-1}) and double bond (1643 cm^{-1}). The ^1H NMR spectrum of **1** (Table 1) indicated the presence of two methyl groups at δ_H 0.99 (3H, s) and 1.02 (3H, d, J = 7.0 Hz), an oxygenated methylene at δ_H 3.43 (1H, d, J = 12.0 Hz) and 3.46 (1H, d, J = 12.0 Hz), and an oxygenated methine at δ_H 4.37 (1H, d, J = 6.5 Hz). The presence of an α,β-unsaturated butenolide system with the double bond located at the junction of two rings was confirmed on the basis of the NMR spectral data: one carbonyl group

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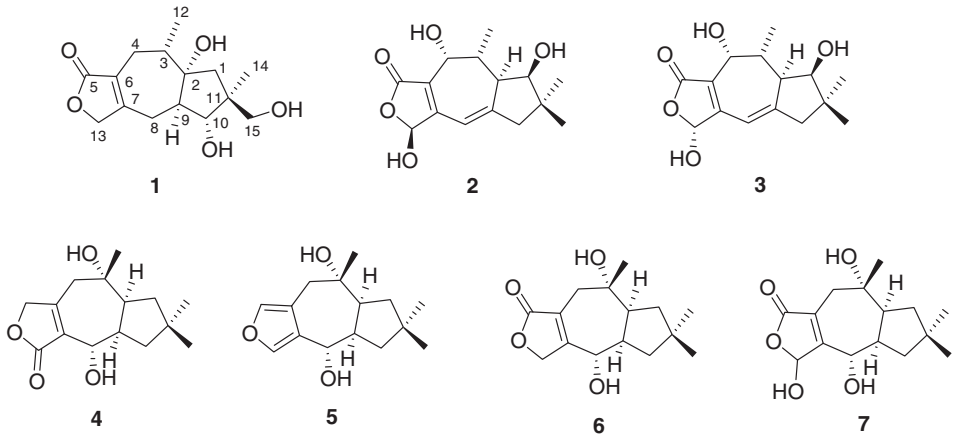

Figure 1. The structures of compounds **1**–**7** isolated from *L. subvellereus*.

(δ_C 176.0), two unsaturated quaternary carbons (δ_C 127.4 and 163.5), and one lactonic methylene (δ_H 4.70, dd and 4.80, d/δ_C 72.0).[10] In addition, the ^{13}C NMR spectrum of **1** with analysis of DEPT spectra showed signals attributed to two methyls (δ_C 15.4 and 20.0), five methylenes with two oxygenated methylenes (δ_C 22.0, 26.9, 47.1, 69.8, and 72.0), three methines with an oxygenated methine (δ_C 39.9, 54.7, and 74.9), two quaternary carbons with an oxygenated carbon (δ_C 44.6 and 81.9), two unsaturated quaternary carbons (δ_C 127.4 and 163.5), and a carbonyl carbon (δ_C 176.0). These data suggested that compound **1** was a sesquiterpene lactone with a lactarane skeleton, with one methyl group substituted to a hydroxyl methylene group.[7,11]

These data and analysis of 2D NMR experiments (^{1}H–^{1}H COSY, HMQC, and HMBC) established the planar structure of compound **1**. The COSY spectrum of **1** allowed for two partial structures, C4–C3–C12 and C8–C9–C10 (Fig. 2). The connectivities between these partial structures were clarified by HMBC: H-8/C-2, H-10/C-2, and H-12/C-2. Based on the correlations H-4/C-5, H-8/C-6, and H-8/C-13 in the HMBC spectrum (Fig. 2), an unsaturated γ-lactone ring should be formed at C-6 and C-7. Furthermore, the presence of a hydroxyl group at C-15 was suggested on the basis of HMBC correlations H-9/C-1, H-14/C-1, H-14/C-15, and H-15/C-10 as well as the molecular formula and the characteristic chemical shift of H-15 (δ_H 3.43 and 3.46).[10]

The absolute configurations of **1** were established on the basis of the convenient Mosher ester procedure combined with important NOESY correlations (Fig. 2).[12] Esterification of **1** yielded the diastereoisomeric MTPA diesters, bis[(*S*)-MTPA] ester (**1s**) and bis[(*R*)-MTPA] ester (**1r**).[13] Diagnostic ^{1}H NMR chemical shift differences between the MTPA esters of **1** [$\Delta\delta = \delta_S - \delta_R$] (Fig. 3) revealed an absolute configuration at C-10 of *R*. This suggested that H-10 has the β orientation. The cross peaks H-10/H-15, H-12/H-14, H-9/H-12, and H-3/H-15 in the NOESY spectrum allowed us to assign the stereochemistries at C-3 and C-11. Furthermore, the very high-field chemical shift of C-12 (δ_C 15.4) in the ^{13}C NMR spectrum indicated that the configuration of hydroxyl group at C-2 is *syn* to the methyl (C-12) as blennin D.[14] Thus, the structure of **1** was established as (10*R*)-2α,10,13,15-tetrahydroxy-lactara-6-en-5-oic acid γ-lactone and the compound named subvellerolactone B.

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of **1**–**3** in CD_3OD (δ in ppm)

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1α	2.02 (d, 14.5)	47.1	4.05 (d, 6.0)	82.5	4.05 (d, 6.0)	82.5
1β	1.74 (d, 14.5)					
2		81.9	2.95 (dd, 11.0, 6.0)	38.8	2.95 (dd, 11.0, 6.0)	38.8
3	2.03 (m)	39.9	1.73 (m)	37.7	1.73 (m)	37.5
4α	2.42 (m)	26.9		66.7		66.5
4β	2.00 (m)		4.42 (br s)		4.38 (br s)	
5		176.0		172.5		172.5
6		127.4		127.5		127.2
7		163.5		154.0		154.0
8	2.35 (m)	22.0	6.31 (br s)	113.1	6.27 (br s)	113.0
	2.66 (m)					
9	2.37 (m)	54.7		168.1		168.1
10α		74.9	1.75 (d, 18.0)	41.3	1.75 (d, 18.0)	41.3
10β	4.37 (d, 6.5)		1.77 (d, 18.0)		1.77 (d, 18.0)	
11		44.6		40.3		40.3
12	1.02 (d, 7.0)	15.4	1.16 (d, 7.0)	15.2	1.16 (d, 7.0)	15.2
13α	4.70 (dd, 17.5, 2.5)	72.0	6.09 (s)	97.6		97.4
13β	4.80 (d, 17.5)				6.02 (s)	
14	0.99 (s)	20.0	1.01 (s)	24.9	1.01 (s)	24.9
15	3.43 (d, 12.0)	69.8	0.99 (s)	21.0	0.99 (s)	21.0
	3.46 (d, 12.0)					

Assignments were based on 2D NMR including HMQC and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hertz (Hz) in parentheses.

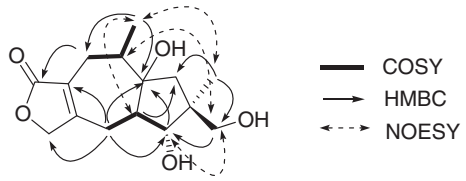

Figure 2. 2D NMR correlations of **1**.

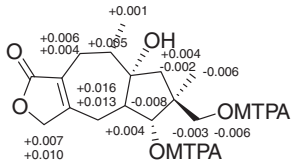

Figure 3. $\Delta\delta$ Values (δ_S–δ_R) in ppm of the two MTPA esters derived from **1**.

Subvellerolactone D (**2**)[15] was isolated as a colorless oil. The molecular formula of **2** was $C_{15}H_{20}O_5$ by positive mode HR-FABMS data at *m*/*z* 303.1211 $[M+Na]^+$ (calcd for $C_{15}H_{20}NaO_5$, 303.1208). Its IR spectrum revealed the absorption bands of a lactonic group at 1739 cm^{-1}, hydroxyl groups at 3384 cm^{-1}, and double bonds at 1645 cm^{-1}. The maximum absorption in the UV spectrum of **2** at 290 nm was attributable to the cross-conjugated system as found in subvellerolactone A.[7] The ^1H NMR spectrum of **2** showed signals of three methyl groups (δ_H 1.16, 1.01, and 0.99) typical of the lactarane skeleton, and one olefinic proton signal (δ_H 6.31). The ^{13}C NMR spectrum indicated four low-field unsaturated carbons (δ_C 113.1, 127.5, 154.0, and 168.1) and a carbonyl carbon (δ_C 172.5). The ^1H and ^{13}C NMR spectra of **2** were similar to those of subvellerolactone A,[7] except for the presence of an additional hydroxyl group (δ_H 4.05/δ_C 82.5). The structure of compound **2** was determined by detailed analysis of 2D NMR experiments (^1H–^1H COSY, HMQC, and HMBC) (Fig. 4). The proton (δ_H 4.42) observed as broad singlet at C-4 suggested that the hydroxyl group at C-4 possessed α-configuration, indicating quasi-axial orientation of H-3 and quasi-equatorial orientation of H-4, which is contrary to that of subvellerolactone A ($^3J_{3,4}$ = 9.3 or 8.4 Hz).[7,14] A detailed analysis of the NOESY correlations H-3/H-15, H-12/H-13, H-1/H-14, and H-12/H-14 (Fig. 4), in combination with proposed biosynthesis of the lactarane skeleton,[14,16] suggested that the relative stereochemistries of **2** were 1R^*, 4R^*, and 13S^*, as shown in Figure 4.

Subvellerolactone E (**3**)[17] was obtained as a colorless oil, and had the molecular formula of $C_{15}H_{20}O_5$, as determined by positive mode HR-FABMS at *m*/*z* 303.1217 $[M+Na]^+$ (calcd for $C_{15}H_{20}NaO_5$, 303.1208). Comparison with the ^1H and ^{13}C NMR data of **2** and **3** revealed that both compounds shared the same planar structure, which was further confirmed by detailed 2D NMR (^1H–^1H COSY, HMQC, and HMBC) analysis of **3**. The obvious differences in the ^1H NMR signals of H-8 and H-13 supported that compound **3** is a C-13 epimer of **2**. The NOESY correlations H-3/H-15, H-3/H-13, H-4/H-13, H-1/H-14, and H-12/H-14 indicated that compounds **2** and **3** have the same stereochemistry except for C-13, contrary to that of **2**. Interestingly, compounds **2** and **3** seem to be interconvertible to each other like subvellerolactone A isolated as an epimeric mixture of hydroxyl group at C-13. Although they were isolated as a single isomer at first, they became soon a mixture just like mutarotation of sugars.

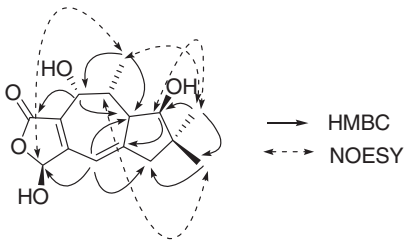

Figure 4. 2D NMR correlations of **2**.

Table 2
Cytotoxicity of compounds **1**–**7** against four cultured human cancer cell lines in the SRB assay

Compound	IC_{50}[a] (μM)			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	26.5	>30.0	18.3	14.2
2	25.1	>30.0	>30.0	17.8
3	19.6	>30.0	>30.0	28.7
4	17.9	>30.0	>30.0	29.3
5	>30.0	>30.0	>30.0	>30.0
6	27.0	>30.0	>30.0	20.9
7	22.9	>30.0	28.3	28.6
Doxorubicin[b]	0.010	0.001	0.002	0.028

[a] 50% inhibitory concentration; the concentration of the compound that caused a 50% inhibition of cell growth.
[b] Doxorubicin as positive control.

Compounds **1**–**7** were evaluated for cytotoxicity against the A549 (non-small cell lung carcinoma), SK-OV-3 (malignant ascites from ovary), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) human tumor cell lines by using the SRB assay.[18] The results (Table 2) showed that compound **1** exhibited cytotoxicity against the A549, SK-MEL-2, and HCT-15 cell lines with IC_{50} values of 26.5, 18.3, and 14.2 μM, respectively, and compounds **2** and **3** showed cytotoxicity against the A549 and HCT-15 cell lines (IC_{50} (**2**): 25.1 and 17.8 μM, and IC_{50} (**3**): 19.6 and 28.7 μM, respectively). The isolated lactarane sesquiterpenes with lactone group (**1**–**4** and **6**–**7**) showed selective cytotoxicity against the A549 and HCT-15 cell lines, yet the lactarane sesquiterpene with furan group (**5**) was inactive against four human tumor cell lines (Table 2).

In conclusion, we focused our investigation on cytotoxic constituents from the fruiting bodies of *L. subvellereus* and found seven lactarane sesquiterpenes (**1**–**7**) including three new cytotoxic lactarane sesquiterpenes, subvellerolactones B (**1**), D (**2**), and E (**3**). The active sesquiterpenes (**1**–**4** and **6**–**7**) could be valuable compounds for future synthetic and pharmacologic studies.

Acknowledgments

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9. *Subvellerolactone B* (**1**): colorless oil; $[\alpha]_D^{25}$ +52.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 218 (3.8) nm; IR (KBr) ν_{max} 3387, 2946, 1768, 1667, 1643, 1455, 1028, 672 cm^{-1}; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; FAB-MS (positive mode) *m*/*z*: 305 $[M+Na]^+$; HRFABMS *m*/*z*: 305.1357 $[M+Na]^+$ (calcd for $C_{15}H_{22}NaO_5$, 305.1365).

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13. Compound **1** (1.5 mg) in deuterated pyridine (0.75 mL) was transferred into a clean NMR tube. (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (10 μL) was added to the NMR tube immediately under a N_2 gas stream, and then the NMR tube was shaken carefully to mix the sample and MTPA chloride evenly. The NMR reaction tube was left at room temperature overnight. The reaction was then completed to afford the bis[(*R*)-MTPA] ester derivative (**1r**) of **1**. The bis[(*S*)-MTPA] ester derivative (**1s**) of **1** was obtained as described for **1r**. The ^{1}H NMR spectra of **1r** and **1s** were measured directly in the NMR reaction tubes. **1s**: ^{1}H NMR (500 MHz, pyridine-d_5) δ 7.999 (2H, m, MTPA-ArH), 7.581 (2H, m, MTPA-ArH), 7.451–7.397 (6H, m, MTPA-ArH), 6.509 (1H, d, *J* = 6.0 Hz, H-10), 4.869 (1H, d, *J* = 12.0 Hz, H-15a), 4.817 (1H, d, *J* = 12.0 Hz, H-15b), 4.729 (1H, d, *J* = 17.5 Hz, H-13a), 4.565 (1H, dd, *J* = 17.5, 2.5 Hz, H-13b), 3.832 (3H, s, OCH_3), 3.561 (3H, s, OCH_3), 2.988 (1H, d, *J* = 14.0 Hz, H-1a), 2.826 (1H, m, 8a), 2.616 (1H, m, H-4a), 2.340 (1H, m, H-9), 2.234 (1H, m, 4b), 2.153 (1H, m, H-3), 2.078 (1H, m, H-8b), 1.885 (1H, d, *J* = 14.0 Hz, H-1b), 1.357 (3H, s, H-14), 1.192 (3H, d, *J* = 7.0 Hz, H-12). **1r**: ^{1}H NMR (500 MHz, pyridine-d_5) δ 7.998 (2H, m, MTPA-ArH), 7.581 (2H, m, MTPA-ArH), 7.450–7.396 (6H, m, MTPA-ArH), 6.517 (1H, d, *J* = 6.0 Hz, H-10), 4.875 (1H, d, *J* = 12.0 Hz, H-15a), 4.820 (1H, d, *J* = 12.0 Hz, H-15b), 4.722 (1H, d, *J* = 17.5 Hz, H-13a), 4.555 (1H, dd, *J* = 17.5, 2.5 Hz, H-13b), 3.833 (3H, s, OCH_3), 3.561 (3H, s, OCH_3), 2.984 (1H, d, *J* = 14.0 Hz, H-1a), 2.813 (1H, m, 8a), 2.610 (1H, m, H-4a), 2.336 (1H, m, H-9), 2.230 (1H, m, 4b), 2.148 (1H, m, H-3), 2.062 (1H, m, H-8b), 1.887 (1H, d, *J* = 14.0 Hz, H-1b), 1.363 (3H, s, H-14), 1.191 (3H, d, *J* = 7.0 Hz, H-12).
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15. *Subvellerolactone C* (**2**): colorless oil; $[\alpha]_D^{25}$ +98.4 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε): 290 (3.2) nm; IR (KBr) ν_{max} 3384, 2949, 2833, 1739, 1665, 1645, 1452, 1033, 672 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; FAB-MS (positive mode) *m*/*z*: 303 [M+Na]$^+$; HRFABMS *m*/*z*: 303.1211 [M+Na]$^+$ (calcd for $C_{15}H_{20}NaO_5$, 303.1208).
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17. *Subvellerolactone D* (**3**): colorless oil; $[\alpha]_D^{25}$ +58.8 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε): 290 (3.2) nm; IR (KBr) ν_{max} 3383, 2949, 2833, 1739, 1666, 1645, 1452, 1033, 672 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; FAB-MS (positive mode) *m*/*z*: 303 [M+Na]$^+$; HRFABMS *m*/*z*: 303.1217 [M+Na]$^+$ (calcd for $C_{15}H_{20}NaO_5$, 303.1208).
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