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ORIGINAL ARTICLE

Three New Fatty Acid Esters from the Mushroom *Boletus pseudocalopus*

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Abstract A bioassay-guided fractionation and chemical investigation of a MeOH extract of the Korean wild mushroom *Boletus pseudocalopus* resulted in the identification of three new fatty acid esters, named calopusins A–C (**1–3**), along with two known fatty acid methyl esters (**4–5**). These new compounds are structurally unique fatty acid esters with a 2,3-butanediol moiety. Their structures were elucidated through 1D- and 2D-NMR spectroscopic data and GC–MS analysis as well as a modified Mosher's method. The new compounds **1–3** showed significant inhibitory activity against the proliferation of the tested cancer cell lines with IC_{50} values in the range 2.77–12.51 μM.

Keywords *Boletus pseudocalopus* · Boletaceae · Fatty acid esters · Cytotoxicity

Abbreviations

SRB	Sulforhodamine B
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HR	High resolution
ESI	Electrospray ionization
EI	Electron-ionization
MS	Mass spectrometry
NMR	Nuclear magnetic resonance

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Introduction

In our continuing search for structurally interesting and bioactive metabolites from Korean wild mushrooms [1–5], we have collected scores of endemic Korean mushroom species in the mountainous areas during the hot humid summer and prepared MeOH extracts of them for antitumor-activity screening tests. Among the collected wild mushrooms, the extract of *Boletus pseudocalopus* (Boletaceae) showed significant cytotoxicity against three human tumor cell lines (A549, Hs746T, and H3122) using a sulforhodamine B (SRB) bioassay [6]. *B. pseudocalopus* is an inedible mushroom with toxicity that is recognized by its yellow fruiting bodies and the blue staining when the fruitbodies are cut or brushed [7], which is presumably due to the presence of the characteristic bolete pigments such as xerocomic acid, variegatic acid, and variegatorubin [8]. The isolation of cytotoxic grifolin derivatives was reported in a recent phytochemical study of this mushroom [8]. Bioassay-guided fractionation and chemical investigation of the MeOH extract of the fruiting bodies of *B. pseudocalopus* resulted in the isolation of three new fatty acid esters (**1–3**) with a 2,3-butanediol moiety, together with two known fatty acid methyl esters (**4–5**) (Fig. 1). In this study, we describe the isolation and structural elucidation of the three new fatty acid esters (**1–3**) from *B. pseudocalopus*, and the cytotoxic activities of the isolates (**1–5**).

Materials and Methods

General Experimental Procedures

Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). IR spectra were

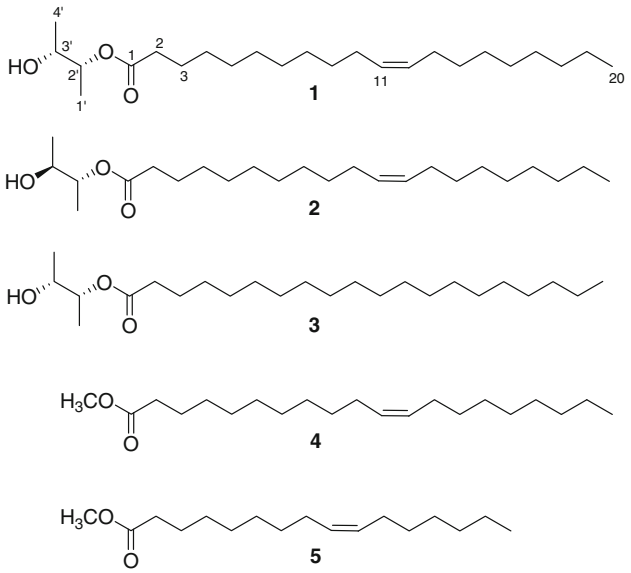


Fig. 1 The structures of the compounds **1–5**

recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). Electrospray ionization (ESI) and high-resolution (HR)-ESI mass spectra were recorded on a SI-2/LCQ DecaXP liquid chromatography (LC)-mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA). Electron-ionization (EI) mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative high performance liquid chromatography (HPLC) used a Gilson 306 pump (Gilson, Middleton, WI, USA) with a Shodex refractive index detector (Shodex, New York, NY, USA). Low-pressure liquid chromatography (LPLC) was carried out over a LiChroprep Lobar-A Si 60 column (240 × 10 mm i.d.; Merck, Darmstadt, Germany) with a FMI QSY-0 pump (Teledyne Isco, Lincoln, NE, USA). Column chromatography was performed with silica gel 60 (Merck, 230–400 mesh). The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Merck precoated silica gel F_{254} plates and reversed-phase (RP)-18 F_{254s} plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

Mushroom Material

The fresh fruiting bodies of *Boletus pseudocalopus* Hongo were collected on Mt. Gaya, Hapcheon-Gun of Gyeongsangnam-do, Korea, in August, 2006. The mushroom was identified by one of the authors (K.R.L.), according to the taxonomic key of Imazeki and Hongo [9]. A voucher specimen (SKKU 2006–08) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation

The air-dried (30–40 °C for 20 h) and powdered fruiting bodies of *B. pseudocalopus* (139 g) were extracted twice with 80% aqueous MeOH (each 1.5 L × 2 day) at room temperature and filtered. The filtrate was evaporated under vacuum to obtain a crude MeOH extract (10 g), which was suspended in distilled water and then successively partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH, yielding 600, 700, and 2,100 mg of residues, respectively. Each fraction was evaluated for cytotoxicity against three human tumor cell lines (A549, Hs746T, and H3122) using an SRB bioassay. We selected the $CHCl_3$-soluble fraction for the current phytochemical study, since the $CHCl_3$-soluble fraction showed significant cytotoxic activity against the tested tumor cell lines. The $CHCl_3$-soluble fraction (700 mg) was separated on a silica gel (230–400 mesh) column chromatography [50 g, 1.5 × 25 cm, eluted with $CHCl_3$–MeOH, 10:1 (1.0 L) and 5:1 (1.0 L)] to yield six fractions [Fr. A, 10:1, 0.3 L; Fr. B, 10:1, 0.3 L; Fr. C, 10:1, 0.4 L; Fr. D, 5:1, 0.3 L; Fr. E, 5:1, 0.3 L; and Fr. F, 5:1, 0.4 L]. Fraction A (350 mg) was subjected to fractionation with Sephadex LH-20 column chromatography [320 g, 3 × 60 cm, eluted with CH_2Cl_2–MeOH, 1:1 (2.0 L)] to give three subfractions [Fr. A1, 1:1, 0.7 L; Fr. A2, 1:1, 0.3 L; and Fr. A3, 1:1, 1.0 L]. Subfraction A2 (150 mg) was applied to LPLC on a LiChroprep Lobar-A Si gel 60 column (240 × 10 mm i.d., 40–63 μm) with a solvent system of $CHCl_3$-MeOH (25:1, 800 mL) to give two fractions (A21–A22). Subfraction A21 (50 mg) was purified by preparative normal-phase HPLC using an Apollo Silica column (Alltech, 250 × 10 mm i.d., 5 μm) with a solvent system of *n*-hexane–EtOAc (15:1, each 800 mL, flow rate; 2 mL/min) to obtain compounds **4** (5 mg) and **5** (7 mg). Subfraction A22 (65 mg) was purified by preparative normal-phase HPLC using an Apollo Silica column (Alltech, 250 × 10 mm i.d., 5 μm) with a solvent system of *n*-hexane–EtOAc (5:1, each 800 mL, flow rate; 2 mL/min) to afford compounds **1** (4 mg), **2** (3 mg), and **3** (4 mg).

*Calopusin A (**1**)*

Colorless gum, $[\alpha]_D^{25}$ +11.6 (*c* 0.12, $CHCl_3$); IR (KBr) ν_{max} 3,355 (OH), 2,943, 1,721 (C=O), 1,280, 1,028 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; ESI–MS (positive mode) *m/z* 405 $[M + Na]^+$; HRESIMS *m/z* 405.3347 $[M + Na]^+$ (calcd. for $C_{24}H_{46}NaO_3$, 405.3345).

Table 1 ^{1}H (500 MHz) and ^{13}C-NMR (125 MHz) data of **1–3** in $CDCl_3$ (δ in ppm)[a]

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		173.7		173.6		173.5
2	2.31 (t, 7.5)	34.8	2.31 (t, 7.5)	34.8	2.31 (t, 7.5)	34.8
3	1.63 (m)	25.2	1.63 (m)	25.2	1.63 (m)	25.2
4	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
5	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
6	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
7	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
8	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
9	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
10	2.01 (m)	27.4	2.01 (m)	27.4	1.26 (br s)	29.6–29.2
11	5.34 (m)	130.2[b]	5.34 (m)	130.2[c]	1.26 (br s)	29.6–29.2
12	5.34 (m)	129.9[b]	5.34 (m)	129.9[c]	1.26 (br s)	29.6–29.2
13	2.01 (m)	27.4	2.01 (m)	27.4	1.26 (br s)	29.6–29.2
14	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
15	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
16	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
17	1.25 (br s)	29.8–29.3	1.25 (br s)	29.6–29.3	1.26 (br s)	29.6–29.2
18	1.27 (m)	31.4	1.27 (m)	32.1	1.27 (m)	32.1
19	1.30 (m)	22.9	1.30 (m)	22.8	1.32 (m)	22.9
20	0.88 (t, 7.0)	14.3	0.88 (t, 7.0)	14.3	0.88 (t, 7.0)	14.3
1′	1.19 (d, 6.5)	16.5	1.18 (d, 6.5)	14.3	1.19 (d, 6.5)	16.5
2′	4.77 (m)	74.8	4.87 (m)	74.3	4.77 (m)	74.7
3′	3.75 (m)	70.3	3.87 (m)	69.8	3.75 (m)	70.3
4′	1.17 (d, 6.5)	19.3	1.16 (d, 6.5)	18.0	1.17 (d, 6.5)	19.3

[a] *J* values are in parentheses and reported in Hz; the assignments were based on HMQC, and HMBC experiments

[b,c] Assignments may be interchangeable

Calopusin B (2)

Colorless gum, $[\alpha]_D^{25}$ −10.7 (*c* 0.14, $CHCl_3$); IR (KBr) ν_{max} 3,356 (OH), 2,943, 1720 (C=O), 1,280, 1,028 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; ESI–MS (positive mode) *m/z* 405 $[M + Na]^+$; HRESIMS *m/z* 405.3353 $[M + Na]^+$ (calcd. for $C_{24}H_{46}NaO_3$, 405.3345).

Calopusin C (3)

Colorless gum, $[\alpha]_D^{25}$ +13.8 (*c* 0.18, $CHCl_3$); IR (KBr) ν_{max} 3,355 (OH), 2,943, 1,721 (C=O), 1,280, 1,028 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; ESI–MS (positive mode) *m/z* 407 $[M + Na]^+$; HRESIMS *m/z* 407.3507 $[M + Na]^+$ (calcd. for $C_{24}H_{48}NaO_3$, 407.3501).

Alkaline Hydrolysis of **1–3**

Hydrolysis of **1–3** (each 1.0 mg) was performed with 5% KOH in MeOH (10 ml) under reflux for 2 h. After cooling, each reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The organic layer was washed with H_2O and filtered. The filtrate was chromatographed by a silica gel Waters Sep-Pak Vac 6 cc (Waters) with a solvent system of *n*-hexane–EtOAc (15:1) to give the fatty acid part. Methyl ester derivatives of fatty acids from **1–3** were prepared by refluxing fatty acids with 1% H_2SO_4 in MeOH (10 ml) for 1 h. The methyl ester mixture was analyzed with GC–MS. The GC–MS analyses were carried out using a Hewlett Packard 6,890–5,973 N GC–MS system operating on electron impact mode (equipped with a HP-5MS UI 60 × 0.25 mm, 0.25 μm film thickness capillary column). Helium (1.5 ml/min) was used as the carrier gas. The initial temperature of the column was 60 °C, and then heated to 280 °C at a rate of 3 °C/min. The identification of the fatty acid esters was based on comparison of their EI-mass spectra with the NIST/NBS, Wiley library spectra. The aqueous layer was neutralized by passage through an Amberlite IRA-67 column (Rohm and Haas) and was repeatedly evaporated to give each 2,3-butanediol moiety.

The 2,3-butanediol from **1** and **3** showed the negative optical rotation, $[\alpha]_D^{25}$ − 16.3 (*c* 0.07, $CHCl_3$) and the ^{1}H-NMR data was consistent with commercial sample (*R*,*R*)-2,3-butanediol. The ^{1}H-NMR data of the 2,3-butanediol, $[\alpha]_D^{25}$ + 10.8 (*c* 0.05, $CHCl_3$) from **2** was consistent with commercial sample, *meso*-2,3-butanediol.

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of **1–3**

To a stirred solution of **1** (1.0 mg) in pyridine (400 μL) was added 4-(dimethylamino)pyridine (1 mg) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl, 10 μL). The mixture was stirred at room temperature for 16 h. The reaction mixture was then passed through a silica gel Waters Sep-Pak Vac 6 cc and eluted with *n*-hexane–EtOAc (15:1) to give the respective (*R*)-Mosher ester **1r**. Treatment of **1** (1.0 mg) with (*R*)-MTPA-Cl (10 μL) as described above yielded the corresponding (*S*)-MTPA ester **1s**. Similarly, treatment of **2** and **3** with (*S*)- and (*R*)-MTPA-Cl afforded the respective Mosher esters **2r**, **2s**, **3r**, and **3s** (Table 2).

Cytotoxicity Testing

A sulforhodamine B (SRB) bioassay was used to determine the cytotoxicity of each compound against four cultured human tumor cell lines [6]. The assays were performed at the Korea Research Institute of Chemical Technology. The cell lines used were A549 (lung carcinoma), Hs756T (stomach carcinoma), H3122 (non-small cell lung carcinoma) and HUVEC (human umbilical cord endothelial cells). The cancer cell lines such as A549, Hs756T, and H3122 cells were provided by the National Cancer Institute (NCI). A normal cell line, HUVEC, was purchased from the American Type Culture Collection. Etoposide (purity ≥98%, Sigma) was used as a positive control. The cytotoxicities of etoposide against the A549, Hs756T, and H3122 cell lines were IC_{50} 0.24, 0.12, and 0.84 μM, respectively. Tested compounds were demonstrated to be pure as evidenced by NMR and HPLC analyses (purity ≥95%).

Results

The fruiting bodies of *B. pseudocalopus* were extracted with 80% aqueous MeOH. The methanolic extract showed cytotoxicity against some human tumor cell lines using a SRB bioassay in our screening procedures. Bioassay-guided fractionation and chemical investigation of the extract using successive column chromatography over silica gel and Sephadex LH-20, and preparative HPLC resulted in the isolation and identification of three new fatty acid esters (**1–3**), together with two known fatty acid methyl esters (**4–5**) (Fig. 1). Their structures were elucidated as follows.

Compound **1** was isolated as a colorless gum. The molecular formula of **1** was determined to be $C_{24}H_{46}O_3$ by positive mode HRESIMS data at *m/z* 405.3347 $[M + Na]^+$ (calcd. for $C_{24}H_{46}NaO_3$, 405.3345). The IR spectrum indicated that **1** possessed hydroxyl (3,355 cm^{-1}) and carbonyl (1,721 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** (Table 1) was similar to that of **4** [10], except that the proton and carbon resonances of a methoxy group in **4** were absent, and the resonances of a 2,3-butanediol moiety at δ_H 4.77 (1H, m, H-2′), 3.75 (1H, m, H-3′), 1.19 (3H, d, J = 6.5 Hz, H-1′), and 1.17 (3H, d, J = 6.5 Hz, H-4′); δ_C 74.8 (C-2′), 70.3 (C-3′), 19.3 (C-4′), and 16.5 (C-1′) were present in **1** [11]. This structure was confirmed by the HMBC spectrum (Fig. 2), where HMBC correlation between H-2′ (δ_H 4.77) and C-1 (δ_C 173.7) indicated the linkage of the 2,3-butanediol unit at C-1. Alkaline hydrolysis of **1** afforded a 2,3-butanediol moiety and a fatty acid unit. The fatty acid unit after esterification with methanol was analyzed as a methyl ester using GC–MS and was identified as methyl 11(*Z*)-eicosenoate (**4**). The double bond position was further confirmed by analysis of the mass fragmentation in the EIMS data (Fig. 2). The absolute configuration of the 2,3-butanediol unit was determined using a modified Mosher's method [12]. Treatment of **1** with (*R*)- and (*S*)-MTPA-Cl gave the (*S*)- and (*R*)-MTPA esters **1s** and **1r**, respectively. The ^{1}H-NMR signals of the two MTPA esters (Table 2) were assigned on the basis of their ^{1}H–^{1}H-COSY spectra, and the $\Delta\delta$ values (δ_S–δ_R) were then calculated (Fig. 3). The results indicated that the

Table 2 Partial ^{1}H-NMR data of the (*S*)- and (*R*)-MTPA esters of **1–3** in $CDCl_3$ (δ in ppm, 500 MHz)

H	**1**			**2**			**3**		
	(*S*)-Isomer	(*R*)-Isomer	$\Delta\delta_{H(S-R)}$	(*S*)-Isomer	(*R*)-Isomer	$\Delta\delta_{H(S-R)}$	(*S*)-Isomer	(*R*)-Isomer	$\Delta\delta_{H(S-R)}$
1′	1.256	1.315	−0.059	1.255	1.247	+0.008	1.249	1.314	−0.065
2′	5.160	5.185	−0.025	5.335	5.324	+0.011	5.168	5.174	−0.006
3′	4.999	4.987	+0.012	5.171	5.152	+0.019	4.993	4.988	+0.005
4′	1.213	1.143	+0.070	1.135	1.142	−0.007	1.221	1.139	+0.082

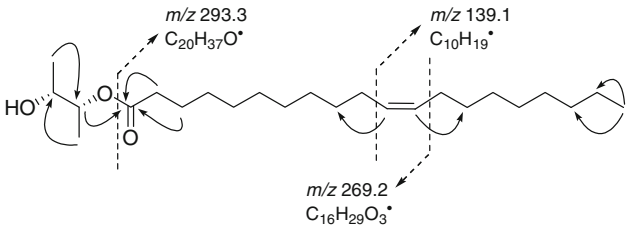


Fig. 2 Key HMBC connectivities and EIMS fragmentation of **1**

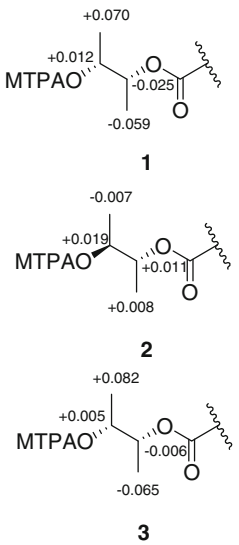
absolute configuration of **1** was 3′*R*. Moreover, the chemical shifts for H-2′ (δ_H 4.77) and H-3′ (δ_H 3.75) suggested that these protons have a *cis*-orientation, which was in agreement with that of the monoacetate of *dl*-butane-2,3-diol [δ_H 4.75 (H-2) and δ_H 3.73 (H-3)] [11] and the 2,3-butanediol obtained by hydrolysis of **1** showed the negative optical rotation, $[\alpha]_D^{25}$ − 16.3 (*c* 0.07, $CHCl_3$), in agreement with that of (*R*,*R*)-2,3-butanediol [13, 14]. Thus, the absolute configuration at C-2′ was confirmed to be *R*. In conclusion, the structure of **1** was elucidated as shown in Fig. 1, and named calopusin A.

Compound **2** was obtained as a colorless gum. The molecular formula of **2** was deduced to be $C_{24}H_{46}O_3$ by positive mode HRESIMS data at *m/z* 405.3353 $[M + Na]^+$ (calcd. for $C_{24}H_{46}NaO_3$, 405.3345). The ^{1}H-NMR data of **2** (Table 1) was very similar to that of **1**, except for the chemical shift and splitting pattern of H-2′ (δ_H 4.87) and H-3′ (δ_H 3.87) in **2** instead of those of H-2′ (δ_H 4.77) and H-3′ (δ_H 3.75) in **1**, which suggested that compound **2** possessed a *meso*-butane-2,3-diol unit on the basis of the chemical shifts of the monoacetate of *meso*-butane-2,3-diol [δ_H 4.86 (H-2) and δ_H 3.88 (H-3)] [11]. This was further supported by the identical ^{1}H-NMR data of the 2,3-butanediol obtained by hydrolysis of **2** to commercial sample, *meso*-2,3-butanediol. Analysis of the HMQC and HMBC correlations led to the establishment of the structure for **2**. As described for **1**, the absolute configuration of **2** was determined using a modified Mosher's method [12], which proved the *S*-configuration for C-3′ (Fig. 3). Then, the absolute configuration of C-2′ was determined to be the *R*-configuration because compound **2** contained a *meso*-butane-2,3-diol unit. Thus, the structure of **2** was assigned as shown in Fig. 1, and named calopusin B.

Compound **3** was isolated as a colorless gum, and had a molecular formula of $C_{24}H_{48}O_3$, as determined by positive mode HRESIMS at *m/z* 407.3507 $[M + Na]^+$ (calcd. for $C_{24}H_{48}NaO_3$, 407.3501). The ^{1}H and ^{13}C-NMR data of **3** (Table 1) were very similar to those of **1**, except for the absence of the chemical shifts attributable to a double bond in **1** [δ_H 5.34 (2H, m, H-11 and H-12); δ_C 130.2 (C-11) and 129.9 (C-12)]. The structure of **3** was confirmed by analysis of 2D-NMR experiments (HMQC and HMBC). As described for **1**, the absolute configuration of **3** was also determined using a modified Mosher's method [12], which proved the *R*-configuration for C-3′ (Fig. 3). Then, the absolute configuration of C-2′ was assigned as *R* on the basis of the identical chemical shifts for H-2′ (δ_H 4.77) and H-3′ (δ_H 3.75) of **3** to those of **1**. Thus, the structure of **3** was determined as shown in Fig. 1, and named calopusin C.

The known fatty acid methyl esters were identified as methyl 11(*Z*)-eicosenoate (**4**) [10] and methyl palmitoleate (**5**) [10] by comparison of their spectroscopic data to previously reported values and analysis of GC–MS.

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



Discussion

The cytotoxic $CHCl_3$-soluble fraction from the MeOH extract of *B. pseudocalopus* was separated by repeated column chromatography to obtain compounds **1–5**. Compounds **1–5** were evaluated for their antiproliferative activities against three human cancer cell lines (A549, Hs756T, and H3122) using the SRB bioassay [6]. Compounds **1–3** showed significant inhibitory activity against proliferation of the tested cancer cell lines with IC_{50} values in the range 2.77–12.51 μM (Table 3). However, the known fatty acid methyl esters (**4–5**) without a 2,3-butanediol group were inactive ($IC_{50} > 20$ μM). In previous studies, the antitumor effect of synthetic 2,3-butanediol fatty acid esters was reported and coixenolide with antitumor activity was isolated from *Coix lacryma-jobi* seeds, which is a mixed ester of palmitoleinic acid and vaccenic acid with the 2,3-butanediol [15, 16]. To confirm whether the 2,3-butanediol unit itself is cytotoxic against the tested cell lines, both of (*R*,*R*)-2,3-butanediol and (*R*,*S*)-2,3-butanediol obtained by hydrolysis of **1–3** were evaluated for cytotoxicity against three human cancer cell lines

Table 3 Cytotoxicity of fractions and compounds **1–5** against a human normal cell line and three human cancer cell lines using SRB bioassay

Fractions and compounds	IC_{50} (μM)[a]			
	A549	Hs746T	H3122	HUVEC
n-hexane-soluble fraction	47.81	37.89	55.43	–
$CHCl_3$-soluble fraction	11.42	6.75	20.15	–
n-BuOH-soluble fraction	63.01	58.92	70.33	–
Fraction A	13.40	5.17	18.62	–
Fraction B	20.86	18.64	17.89	–
Fraction C	32.34	27.47	33.07	–
Fraction D	34.20	40.81	47.90	–
Fraction E	38.71	37.19	48.32	–
Fraction F	26.18	19.54	40.52	–
Fraction A1	18.60	9.45	23.79	–
Fraction A2	10.36	6.28	15.34	–
Fraction A3	21.15	12.42	18.67	–
Fraction A21	15.77	11.16	18.81	–
Fraction A22	7.74	4.82	13.74	–
1	4.97	3.65	12.51	30.97
2	6.02	5.37	9.07	42.92
3	4.04	2.77	8.26	41.71
4	35.30	24.41	66.16	55.27
5	44.59	29.65	60.06	59.59
Etoposide[b]	0.24	0.12	0.84	1.86
Gemcitabine[c]	0.01	0.09	0.01	3.12

[a] IC_{50} value of compounds against each tested cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth in vitro

[b] Etoposide as a positive control

[c] Gemcitabine as a reference compound

(A549, Hs756T, and H3122). But they were inactive ($IC_{50} > 20$ μM). These results demonstrate that though the 2,3-butanediol itself is inactive, it improves antitumor activity in such fatty acid esters. To establish whether cytotoxicity exhibited by compounds **1–3** was selective between tumor and normal cells, these compounds were tested against a normal human cell line, HUVEC. The results (Table 3) showed that **1–3** were more cytotoxic against tumor cells than normal cells, indicating that **1–3** exhibited selective toxicity. In particular, compound **3** showed the highest selective cytotoxicity for Hs746T cell line since it exhibited the greatest selectivity index (SI) value of 15.1. The SI value was obtained by dividing the IC_{50} value for the normal cell line by the IC_{50} value for the tumor cell line.

In conclusion, we focused our investigation on cytotoxic constituents from the fruiting bodies of *B. pseudocalopus* and identified five fatty acid esters including three new compounds, calopusins A-C (**1–3**) showing significant cytotoxicity. Calopusins A-C (**1–3**), which displayed high selective toxicity against the A549, Hs746T, and H3122 cell lines, may be especially promising for developing an effective drug for lung and stomach cancer in this regard.

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Conflict of interest All authors declare that there are no conflicts of interest.

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