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Structure of Three New Terpenoids, Spiciformisins *a* and *b*, and Monocyclosqualene, Isolated from the Herbs of *Ligularia fischeri* var. *spiciformis* and Cytotoxicity

Kyung-Tae Lee[1], Sung-Ja Koo[2], Seung-Hee Jung[2], Jongwon Choi[3], Hyun-Ju Jung[4], and Hee-Juhn Park[4]

[1]College of Pharmacy, [2]Deaprtment of Food and Nutrition, Kyung-Hee University, Seoul, 130-701 Korea, [3]College of Pharmacy, Kyungsung University, Pusan, 608-736, and [4]Division of Applied Plant Sciences, Sangji University, Wonju 220-702, Korea

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The diethylether fraction from the leaves extract of *Ligularia fischeri* var. *spiciformis* (Compositae) was subjected to silica gel column chromatography and yielded three new terpenoids named spiciformisin *a* (**1**), spiciformisin *b* (**3**), and monocyclosqualene (**2**). Acyclic diterpenes, spiciformisin *a* and -*b*, were established as 3,7,11,15-tetramethyl-1,3(20)-hexadecadiene and 3,7,11,15-tetramethyl-1,3,6,10,14-hexadecapentaene (IUPAC), respectively. A monocyclic triterpene, monocyclosqualene, were determined as [3,8,12,16,16-pentamethyl-(3,7,11,15-hexadecatetraenyl)]-3,3,5-trimethyl-1-cyclohexene. The structures were determined on the basis of NMR and MS analysis. Spiciformicin b showed potent cytotoxicity (IC_{50}, <9.7 μg/ml against HL-60) in contrast to no cytotoxicity (IC_{50}, >200 μg/ml against HL-60 cells) of spiciformicin a with a *cis*-conjugated dienyl diexomethylene.

Key words: *Ligularia fischeri* var *spiciformis*, Compositae, Terpenoids, Spciformisin, Monocyclosqualene, Cytotoxicity

INTRODUCTION

The plant of *Ligularia fischeri* (Ledebour) Turcz. var. *spiciformis* Nakai (Compositae) is used for edible herbs as an endemic perrenial herbal species in Korea (Nakai *et al.*, 1943). This vegetable has been also known to be effective in the diseases of jaundice, scarlet fever, rheumatoid arthritis and hepatic function failure (Choi *et al.*, 1991). Ligularia species, in general, contains sesquiterpenes (Zhao *et al.*, 1994; Gao *et al.*, 1997; Jia *et al.*, 1993; Bohlmann 1980), phenylpropanoids (Zhao and Ji *et al.*, 1994; Ma *et al.*, 1997) and pyrrolizidine-type alkaloids (Asada *et al.*, 1984). We have previously reported the isolation of intermedeol and 6-oxoeremophilenolide from diethyl ether fraction and the cytotoxicity of intermedeol and diethylether fraction (Park *et al.*, 2000). We have also reported the inductive effect of intermedeol on HL-60 cell differentiation and its biochemical mechanism (Jeong *et al.*, 2002). Further isolation was undertaken to search for other cytotoxic terpenoids from the diethyl ether fraction with cytotoxicity. This communication presents the isolation of three new terpenoids including two acyclic diterpenes (**1**, **3**) and a monocyclic triterpene, monocylosqualene (**2**) and the structure-cytotoxicity relationship.

Correspondence to: Hee-Juhn Park, Division of Applied Plant Science, Sangji University, 660, Woosan-dong, Wonju 220-702, Korea
E-mail: hjpark@mail.sangji.ac.kr

MATERIALS AND METODS

General experimental procedure

IR spectra were recorded were recorded on a Hitachi 260-01 spectrometer in KBr cells (neat). EI-MS (ionization voltage, 70 eV) was measured with JEOL JMS DX-300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with TMS as an internal standard. 2D NMR spectra (^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and HMBC) were measured by the use of JEOL standard pulse sequences.

Plant material

The leaves of *Ligularia fischeri* var. *spiciformis* were collected in July 1999, in Pyongchang, Kangwondo, South Korea, and the plant was identified by Dr. K. O. Yoo

(Department of Biology, Kangwon National University, Chuncheon, Korea). A voucher specimen (KW-980814) is deposited in the herbarium of Department of Biology, Kangwon National University. This plant was air-dried avoiding sunlight and pulverized for the experiment.

Extraction, fractionantion and isolation

The plant material (2.0 kg) was extracted three times with MeOH under reflux for 5 h and the filtered extract was evaporated on a rotatory evaporator under reduced pressure to give viscous MeOH extract (526 g). This was partitioned between H_2O and diethyl ether. The diethyl ether-soluble part was evaporated *in vacuo* to give diethyl ether fraction (32 g). A part of that fraction (12 g) was subjected to column chromatography over silica gel (350 g, 6×60 cm, Merck, Art. No. 7734, Germany) using the mixed solvent n-hexane-ethylacetate (10 : 1, 5.4 L). The eluents were collected by 60 ml each and checked by spraying vanillin-sulfuric acid reagent on TLC. Then the fractions were divided into 10 fractions. The eluate volume of each fraction is as follows: fraction 1 (fr. 1, 180 ml), fr. 2 (120 ml), fr. 3 (360 ml), fr 4 (480 ml), fr. 5 (1000 ml), fr. 6 (540 ml), fr. 7 (240 ml), fr 8 (240 ml), fr. 9 (300 ml), fr. 10 (180 ml), fr. 11 (600 ml). Repeated column chromatography of fr. 1, fr. 3 and fr. 5 by the same eluting solvent yielded colorless oils of compound **1** (36 mg), **2** (50 mg) and **3** (53 mg), respectively. Repeated column chromatography of fr. 6, fr. 8 and fr. 11 gave compounds **4** (30 mg), **5** (80 mg), and **6** (32 mg). Compounds **4-6** were identified as known, 6-oxoeremophilenolide, intermedeol and β-sitosterol, respectively, compared with NMR and IR spectra of authentic specimens.

Compound **1**. Colorless oil, IR (KBr): ν_{max} = 3064 (=C-H), 2964 (C-H), 1644 cm^{-1} (C=C); ^1H NMR (500 MHz, $CDCl_3$+DMSO-d_6) δ: 0.77 (3H×2, d, H-18,19), 0.79 (3H×2, d, *J* = 4.1 Hz, H-16,17), 0.97-1.01 (6H, m, H-6,10,14), 1.04-1.08 (4H, m, H-8,12), 1.12-1.20 (2H, m, H-9), 1.33 (1H×2, m, H-7, 11), 1.39 (2H, m, H-5), 1.44 (1H, m, H-15), 2.08 (2H, m, H-4), 4.89 (1H, brs, H-20a), 4.90 (1H, d, H-20b), 4.96 (1H, d, *J* = 10.8, H-1a), 5.13 (1H, d, H-1b, *J* = 17.6), 6.25 (1H, dd, *J* = 10.8, 17.6, H-2); ^{13}C NMR (125 MHz, $CDCl_3$+DMSO-d_6) δ: 20.5 (C-18,19), 23.3 (C-16,17), 24.9 (C-9), 25.3 (C-13), 26.1 (C-5), 32.3 (C-4), 33.1 (C-7,11), 37.4 (C-12), 37.8 (C-6), 37.9 (C-8, 10), 40.0 (C-14), 113.8 (C-20), 116.2 (C-1), 139.7 (C-2), 147.9 (C-3); EI-MS (70 eV) m/z (rel. int., %): 278 (M^+, $[C_{20}H_{38}]^+$, 3), 137 (15), 125 (22), 109 (44), 95 (52), 82 (100).

Compound **2**. Colorless oil, IR (KBr): ν_{max} = 3063 (=C-H), 2965 (C-H), 1644 cm^{-1} (C=C); ^1H NMR (500 MHz, $CDCl_3$) δ: 0.90 (3H, d, *J* = 6.8 Hz, H-25), 0.95, 0.98×2 (each 3H, s, H-26,27,28), 1.03, 1.23 (each 3H, s, H-23, 24), 1.59 (1H, m, H-10), 1.64, 1.76 (each 3H, s, H-29,30), 4.72, 4.75, 4.85, 4.97 (each 1H, brs, H-9,13,17,21); 5.34-5.36 (2H, m, H-2,3); ^{13}C NMR (125 MHz, $CDCl_3$) δ: methyls-16.7, 16.9, 20.9, 23.3 (CH_3-25,26,27,28), 26.1 (C-23), 27.8 (C-24), 29.0 (C-29), 29.1 (C-30); 30.4, 30.5, 30.7, 35.4, 37.6, 39.2, 40.4, 40.6, 41.0; methine-38.7 (C-10), 49.1 (C-5); quaternary carbon-54.3 (C-4); olefins-108.8, 108.9, 112.3, 121.1, 124.9, 125.2, 136.1, 144.6, 150.6, 155.3 (olefinic carbons); HRFABMS: m/z 410.3891 (cald for $C_{30}H_{50}$, 410.3915).

Compound **3**. Colorless oil, IR (KBr): ν_{max} = 3066 (=C-H), 2967 (C-H), 1642 cm^{-1} (C=C); ^1H NMR (500 MHz, $CDCl_3$ +DMSO-d_6) δ: 1.61 (3H×2, s, H-18,19), 1.64 (3H, s, H-20), 1.69 (3H×2, s, H-16,17), 2.00 (2H×2, t, *J* = 7.6, H-9,13), 2.07 (2H×2, t, *J* = 7.6 Hz, H-8,12), 2.85 (2H, m, H-5), 5.05-5.15 (1H×3, t-like, H-6, 10,14), 4.93 (1H, d, *J* = 10.7, H-1a), 5.21 (1H, d, *J* = 17.3 Hz, H-1b), 5.35 (1H, t, *J* = 5.4 Hz, H-4), 6.38 (1H, dd, *J* = 10.7, 17.3 Hz, H-2); ^{13}C NMR (125 MHz, $CDCl_3$+DMSO-d_6) δ: 12.3 (C-20), 16.7 (C-17), 18.3 (C-18,19), 26.3 (C-16), 27.0 (C-9), 27.9 (C-9,13), 40.3 (C-8,12), 114.1 (C-1), 122.7 (C-10), 123.0 (C-6), 124.9 (C-14), 130.4 (C-4), 132.6 (C-11), 134.3 (C-3,7,15), 142.2 (C-2); EI-MS (70 eV) m/z (rel. int., %): 272 (M^+, $[C_{20}H_{32}]^+$, 2), 218 (37), 161 (61), 119 (75), 105 (91), 63 (100).

Cytotoxicity assay

The *in vitro* cytotoxic test against HL-60 (human acute promyelocytic leukemia ceel), U-937 (human histocytic leukemia ceel), 3 LL (human carcinoma cell) cells were essentially according to the method previously described (Denizot *et al.*, 1996). Cells (1×10^4) were seeded in each well containing 100 μl of RPMI medium supplemented with 10 % FBS in a 96-well microtiter plate and incubated overnight. The test samples, compounds **1-3** were dissolved in dimethylsulfoxide (DMSO) and were added in serial dilution (the final DMSO concentrations in all assays did not exceed 0.01%). Twenty-four hours after seeding, 100 μl new media or test samples were added, and the plates were incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue which formed in the cells was replaced by adding 50 μl DMSO. Optical density was measured at 540 nm. Cisplatin was used as a positive control.

RESULTS AND DISCUSSION

The phytochemical isolation from diethyl ether extract led to the isolation of compound **1**, **2** and **3** (each colorless oil) together with the known compounds **4** (6-oxoeremophilenolide), **5** (intermedeol) and **6** (sterol). Structures of **1**, **2** and **3** were shown in Fig. 1 (*Notice*: numberings are different from the IUPACs and NMR explanations followed the numbering in Fig. 1). The IR spectrum of **1**

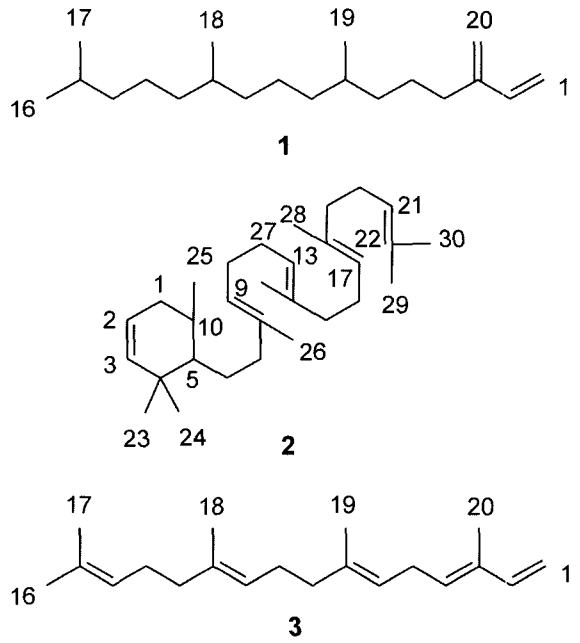

Fig. 1. Chemical strucutre of compounds **1** (spiciformsin *a*), **3** (spiciformisin b) and **2** (monocyclosqualene) isolated from *Ligularia fischeri* var. *spiciformis*.

showed alkenyl C-H at 3064 cm^{-1} and aliphatic C=C at 1644 cm^{-1} but no OH band. In the ^{1}H-NMR spectrum of compound **1**, four doublet methyl peaks were shown at δ 0.77 (3H×2) and 0.79 (3H×2) and five olefinic protons at δ 4.89 (brs), 4.90 (brs), 4.89 (brs), 4.96 (d, *J* = 10.8 Hz), 6.25 (dd, *J* = 10.8, 17.6 Hz), respectively. Four olefinic protons besides a peak at δ 6.25 indicated that his compound has the two exomethylenes. The coupling constants of a double doublet peak at δ 6.25 suggested that a proton at C-2 magnetically couples with the two protons at C-1 in the ^{1}H-NMR. The carbon signals of exomethylene in **1** were also shown at δ 113.8 (C-20), and 116.2 (C-1), and the two signals at δ 147.9 (C-3) and 139.7 (C-2) were observed aided by the ^{1}H-^{13}C COSY NMR spectrum. Other peaks were compared with those of phytol (Sims *et al.*, 1976). The ^{1}H- and ^{13}C NMR spectra confirmed that **1** must be an acyclic diterpene. Thus, the structure of compound **1** was determined as 6,7,10,11,14,15-hexahydro-β-springene (IUPAC: 3,7,11,15-tetramethyl-1,3(20)-hexadecadiene), which was named spiciformisin *a*.

The molecular composition of compound **2** ($C_{30}H_{50}$) was established by HRFABMS indicating one of squalene series. The IR spectrum of compound **2** showed no functional band other than aliphatic C-H and olefinic C=C, which were consistent with compound **1**. In the ^{1}H-NMR spectrum of **2**, seven singlet methyl signals between δ 0.90-1.76 and one doublet methyl signal at 0.90 (d, *J* = 6.8 Hz) together with 6 olefinic protons at δ 4.72, 4.75, 4.85, 4.97, 5.35, 5.36 were found. Since all 30 carbon signals were found in the ^{13}C NMR spectrum, this data indicated that compound **2** is a squalene derivative belonging to triterpenes. In addition, it was found that **2** had 8 methyls, 9 methylenes, 2 methines, a quaternary carbon and 10 olefines (totally 30 carbon) on the basis of ^{13}C-NMR and DEPT NMR spectrum. The doublet methyl signal at δ_H 0.90 were interpreted that compound **1** has secondary methyl at C-10. The structure with a cyclic ring which was shown in Fig. 1 can be supported by the appearance of δ_C 54.3 (C-4) and 49.1 (C-5). On the HMBC spectrum, it was shown that H-23 (δ 1.03) and H24 (δ 1.23) correlated with carbons of a double bond (δ_C 150.6 and 155.3) confirming that this double bond situated between C-2 and C3. Thus, compound **2** was found as monocyclosaqualene (IUPAC: [3,8,12,16,16-pentamethyl-(3,7,11,15-hexadecatetraenyl)]-3,3,5-trimethyl-1-cyclohexene).

The IR spectrum of compound **3** also showed olefinic band at 1642 cm^{-1} but no other functional groups such as OH and C=O. In the ^{1}H NMR spectrum, five singlet methyl peaks δ 1.61 (H-18,19), 1.64 (H-20), 1.69 (H-16,17), and three olefinic protons {δ 5.21 (*J* = 17.3, H-1b), δ 4.93 (*J* = 10.7, H-1a), δ 6.38 (*J* = 10.7, 17.3, H-2)} coupled one another were found, respectively. Among other 4 olefinic protons, the three were shown overlapped at δ 5.05-5.15 and one was shown at δ 5.35 (t, *J* = 5.4 Hz). Unlike in the ^{1}H NMR spectrum of **1**, compound **3** does not have four olefinic proton due to two exomethylene groups but only three protons indicating that **3** has one exomethylene conjugated with the other olefin. In the ^{13}C-NMR spectrum, the signals due to a conjugated double bond with one exomethylene were found at δ 114.1 (C-1), 142.2 (C-2), 134.3 (C-3), 130.4 (C-4). Other peaks were assigned compared with those of β-springene (Atta-ur-Rahman *et al.*, 1978). Thus, the structure of compound **3** was determined as 4-dehydro-17-hydro-β-springene (IUPAC: 3,7,11,15-tetramethyl-1,3,6,10,14-hexadecapentaene) and it was named spiciformisin *b*.

We have reported the cytotoxicity of diethyl ether fraction and its component, intermedeol, from this plant, which may be dependent on the exomethylene in that compound (Park *et al.*, 2000). Among additional terpenoids, spiciformisin *a* (**1**), monocyclosqualene (**2**) and spiciformisin *b* (**3**) were isolated from the ether fraction of *L. fischerii* var. *spiciformis*, and then the two acyclic diterpenes, **1** and **3** were tested on MTT assay for the cytotoxicity. Spiciformicin *b* (**3**) with a partial structure of *trans*-conjugated dienyl exomethylene showed potent cytotoxicity (IC_{50}, <9.7 μg/ml against HL-60; 21.0 μg/ml against 3 LL; 35.6 μg/ml against U-937) in contrast to no cytotoxicity (IC_{50}, >200 μg/ml against the three cancer cells) of spiciformicin a (**1**) with a *cis*-conjugated dienyl diexomethylene. Of spiciformisins, both acyclic diterpenes with each of conjugated dienyl groups, only spiciformisin *b* showed significant

cytotoxicities against cancer cells.

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