Isolation and Biological Activity of Sesquiterpene Lactone

Myung Ju Kim[1], Hyun Ju Oh[2], Seung Hwa Baek*

Department of Herbal Resources, Professional Graduate School of Oriental Medicine and Institute of Basic Natural Sciences, Wonkwang University,
1:Department of Skin & Beauty, Kwangju Health Science College, 2:Department of Beauty & Art, Honam University

Sequiterpene lactone (**1**) has determined by spectroscopic analysis. This compound (**1**) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* (ATCC 28185, 2 mm zone at 15 μg/disk), cytotoxic to murine leukaemia cell lines ATCC CCL 46 P388D1, (IC_{50} 1,125 ng/mL at 7.5 μg/disk) and BSC monkey kidney cell lines (100% of well at 15 μg/disk).

Key words : Sequiterpene lactone (1), *Trichophyton mentagrophytes*, P388, Cytotoxic activity

Introduction

Liverworts are generally located in damp forested areas, growing on trees, rotting logs and rocks or on the ground. Frequently, several species of liverwort will be found growing intertwined, and some species grow to resemble a carpet covering large areas of the forest floor. In New Zealand, liverworts can be found throughout the country, generally in rainforest areas, growing on the ground, or on rotting logs in damp and humid regions[1].

Hepatostolonophora paucistipula(Rodw.) J.J. Engel (family Geocalycaceae) is a rich source of sesquiterpenes in the New Zealand liverworts[2]. In this study, the antiviral and antimicrobial activities and cytotoxicity of *(-)-ent*-arbusculin B (1) from *H. paucistipula* have investigated, and its structure has determined by spectroscopic analysis.

Materials and Methods

1. General experimental procedures

All solvents were distilled before use. Removal of solvents from chromatography fractions were removed by rotary evaporation at temperature up to 40℃. Initial fractionation of crude plant extract using reverse phase column chromatography was performed with octadecyl-functionalized silica gel (C-18 Aldrich) as the adsorbent. Further column fractionation was performed using Davisil silica 60 Å (35-70 ㎛ silica gel, Allth) as adsorbent. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254} visualized first with a UV lamp, then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) followed by heating. Microanalyses were performed by Marianne Dick and Bob McAllister(Campbel Microanalytical Laboratory, Chemistry Department, University of Otago). MS, UVand IR spetra were recorded on Krato MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FT-IR instruments respectively. NMR spectra, of $CDCl_3$ solutions at 25℃, were recorded at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR on a Varian VXR-300 spectrometer. Chemical shifts are given in parts per million on the δ scale referenced to the solvent peak $CHCl_3$ at 7.25 ppm and $CDCl_3$ at 77.08 ppm and are referenced to TMS at 0.00 ppm.

2. Plant material

Hepatostolonophora paucistipula (*H. paucistipula*) was collected from Port Adventure, Stewart Island, in January 1994. This ws identified by D. Glenny, Landcare Research, and a voucher specimen, OTA 046764, has been kept in the Otago University herbarium.

3. Isolation of (-)-ent-arbusculin B

Air-dried *H. paucistipula* (76.3 g) was ground and macrated in redistilled ethanol (1,000 mL) in a Waring Blender, and then filtered. the residual marc was reextracted in the same way with more ethanol (3 x 300 mL). The combined filtrates were evaporated under reduced pressure to give a crude extract (1.585 g). A sub-sample (0.836 g) was fractionated over a C_{18}-bonded silica column (10 g), developed with H_2O, 3

* To whom correspondence should be addressed : Seung Hwa Baek, Department of Herbal Resources, Professional Graduate School of Oriental Medicine, and Institute of Natural Sciences, Wonkwang University

· E-mail : shbaek@wonkwang.ac.kr, · Tel : 063-850-6225

· Received : 2005/07/18 · Revised : 2005/08/29 · Accepted : 2005/09/16

: 1, 1 : 1, and 1 : 3 mixtures of H_2O : CH_3CN, then 1 : 1 - CH_3CN : $CHCl_3$. then $CHCl_3$, then Hexane, then extra $CHCl_3$ and CH_3CN (2 x 17 mL fractions for each solvent mixture). Most of the antimicrobial activity was found in fractions eluted with 1 : 3 - H_2O : CH_3CN (34 mL). This material (96 mg) was fractionated on a silica gel column (1.0 g), developed with 100% hexane (4 mL), 2% (3 x 4 mL), 5% (6 x 4 mL), 10% (5 x 4 mL), 15% (4 x 4 mL), 20% (10 x 4 mL), 30% (3 x 4 mL), 40% (6 x 4 mL), 50% (5 x 4 mL), 75% (4 x 4 mL) mixture of ethyl acetate : hexane, then 100% ethyl actate, then 100% EtOH (5 x 4 mL). Fractions eluted with 5% ehyl acetate : hexane (3 x 4 mL) were combined and yielded white crystals (6.5 mg, P 388 IC_{50} 1492 ng/mL). The second P 388-active fraction from this column was subjected to preparative Si gel TLC (2 : 8 ethyl acetate : hexane) to give (-)-arbusculin B (**1**, 4.3 mg): $[\alpha]^{24.9}$ -18.0 (c, 0.27, hexane); silica TLC R_F 0.56 (2 : 8 - ethyl acetate : hexane); ^1H-NMR ($CDCl_3$): δ 6.14 (1H, d, J=3.2 Hz, 13-H), 5.45 (1H, d=3.0 Hz, 13'-H), 4.55 (1H, dd, J=1.4, 9.8 Hz, 6-H), 2.59 (1H, m, 7-H), 1.87 (3H, brs, CH_3), 1.12 (3H, brs, CH_3); ^{13}C-NMR ($CDCl_3$): δ 170.5 (C-12), 139.54 (C-4), 129.90 (C-5), 126.96 (C-11), 118.12 (C-13), 83.50 (C-6), 50.31 (C-7), 41.30 (C-1), 40.75 (C-2), 37.23 (C-10), 34.40 (C-3), 26.20 (C-15), 23.27(C-8), 19.97 (C-14), 18.84 (C-9); EL-MS (70 eV): 234.1623 (12%, M^+, $C_{15}H_{22}O_2$ requires 234.1620), 232 (29%, M^+-H_2), 217 (100%, M^+-CH_5)[3,4].

4. Disk diffusion assays. Screening for antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested[5]. multiresistant *Bacillus subtilis* (ATCC 19659), *Staphylococcus aureus* (ATCC 6538P) *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 14053). Extracts were dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 5 mg/mL. Test plates are prepared from Mueller Hinton agar containing extract to give a final concentration of 100 μg extract/mL agar. Activity growing cultures of the test strains were diluted in saline so as to deliver 104 colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculator. Inoculated plates were incubated overnight at 37℃. Growth on the blank and control plates was checked and, if satisfactory, growth on the test plates was scored for each test strain as follows: (-) inhibition, no reduction in growth compared with the control, (+) inhibition, no growth. Solutions of compound for assay were dried onto 6 mm filter paper disks, which were then placed onto seeded agar Petri dishes and incubated. Activity was observed as a zone of inhibition around the disk, with its width recorded from the edge of the disk in mm. HM and SM refer to the observed margin surrounding the zone of inhibition. (H= hazy, S= sharp).

5. Cytotoxicity and antiviral assays

Antiviral (*Herpes simplex. Polio*) and antitumor (P388) biological assays were performed by Gill Ellis at the Chemistry Department, University of Canterbury, Christchurch[5]. For the antiviral assay, the samples were dreid onto 8 mm filter paper disks and placed directly onto BSC-1 cells (African Green Monkey kidney), which were then infected with either *Herpes simplex* Type I *virus* (ATCC VR 733) or *Polio* Type I *virus* (Pfizer vaccine strain). After incubation for 24 hours, the cells were examined using an inverted microscope. Two results were obtained: the proportion of cells around the disk that did not show the cytopathic effect of the viruses, and the propotion of cells around the disk that showed a cytotoxic effect. For the P388 assay a two-fold dilution series of the sample was incubated for 72 hours with murine leukaemia cell (ATCC CCL 46 P388D1). The ocncentration of the sample required to inhibit cell growth to 50% of a solvent control was determined using the absorbance obtained upon staining with MTT tetrazolium. Mytomycin C (concentration 0.075 μg/mL) was used as a positive control and inhibited the growth of P388 cells by 43 - 75%.

Results and Discussion

1. Isolation and identification of (-)-*ent*-arbusculin B (1)

Liverworts have been a rich source of sesquiterpenes, including several new skeletal types[2]. When the same compounds have been isolated from liverworts and vascular plants, they are often enantiomeric[2,5]. On the other hand, both enantiomers of some sesquiterpene lactones have been reprted from related liverwort species[2]. For example, (-)-*ent*-arbusculin B (1) has been isolated from *Frullania diltata* and *F. usamiensis*[3,4] and (+)-arbusculin B (2) from *F. serratta* and *F. muscicola*[6,7]. A sub-sample of the extract was subjected to reverse-phase (C-18) silica gel column chromatography. The column fractions were combined based on visually similar TLC results. These combined fractions were assayed against murine leukaemia cell lines (ATCC CCL 46 P388D1) and the activity was found to be spread over six fractions that were eluted with 1 : 1 H_2O/MeCN, 1 : 3 H_2O/MeCN, MeCN, 1 : 1 MeCN/$CHCl_3$, Hex/$CHCl_3$ and MeCN[8]. Because of this, the fraction 4 chromatographed on a silica gel column using a ethyl acetate - hexane gradient. The fraction 4-2 with high activity was shown by TLC and 1H-NMR spectrum to consist of one main UV-active compound. Thin-layer chromatography

spread cytotoxic activity across ethyl acetate - hexane 20 : 80 (R_f 0.56) band containing (-)-ent-arbusculin B (**1**). The MS supported a molecular of $C_{15}H_{22}O_2$. The ^{1}H-NMR spectrum of 1 showed the presence of olefinic group with signals at δ5.45 (1H, d, J= 3.0 Hz) and δ6.14 (1H, d, J= 3.2 Hz) and the protons at C-6 in a allylic bond as one-proton double doublet at δ4.55 (J= 1.4, 9.8 Hz) together with two methyl groups at δ1.12 and 1.87[7].

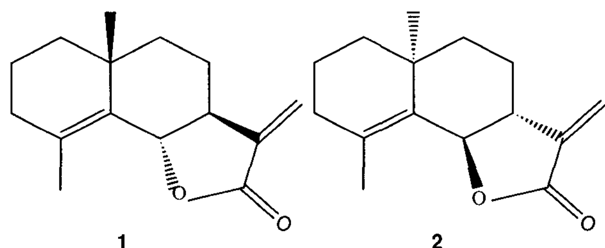

Fig. 1. The structures of (-)-*ent*-arbusculin B(**1**) and (+)-arbusculin B(**2**)

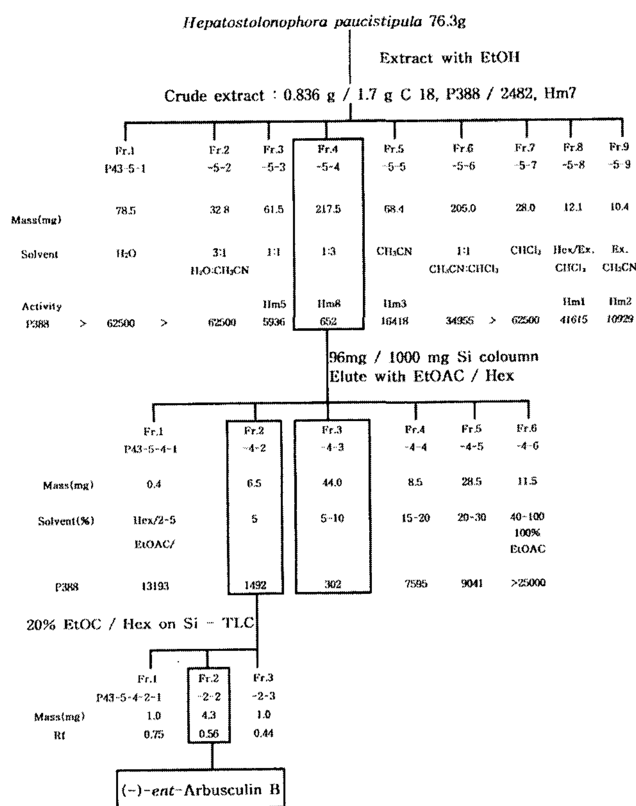

Fig. 2. Isolation of (-)-*ent*-arbusculin B (**1**) from *H. paucistipula.*

2. Biological activities of (-)-*ent*-arbusculin B (**1**).

(-)-*ent*-Arbusculin B (**1**) from *H. paucistipula* has known as (-)-Y-cyclocostunolide isolated from two *Frullania* species of liverwort[7]. This compound (**1**) is cytotoxic to murine leukaemia cells ATCC CCL 46 P388D1, (1,125 ng/mL at 7.5 μg/disk) and BSC monkey kidney cells (100% activity, at 15 μg/disk). Table 1 shows the strong antiviral activity against *Herpes simplex* Type I *virus* (ATCC VR 733) and *Polio* Type I *virus* (Pfizer vaccine strain) (100% activity, @ 0.5 mg/mL at 15 μg/disk). As indicated in Table 1, the compound (**1**) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (2 mm inhibition zone at 15 μg/disk). The activities are expressed by the diameter of the developed inhibition zones and compared with those of the widely antibious chloramphenicol, gentamycin and nystatin (Table 1)[9].

Table 1. Biological activities of (-)-*ent*-arbusculin B (1) from *H. paucistipula*

Assay	Tested material				
	1	Chloramphenicol	Nystatin	Gentamycin	Mitomycin C
Cytotoxicity					
BSC-1 cells[a]	+ + + +				
P388					
IC_{50}	1,125[b]				56.4[c]
Antiviral activity[d]					
Herpes simplex virus	+ + + +				
Polio virus	+ + + +				
Antimicrobial activity[e]					
B. subtilis	-	SM 12	0		0
E. coli	-	0	0		SM 9
P. aeruginosa	-	0	0		SM 10
C. albicans	-	0	SM 11		0
C. resinae	-	0	SM 10		0
T. mentagrophytes	HM 2	0	HM 8		0

[a]% of well showing cytotoxic effects. @ 0.5 mg/mL, 15 μg/disk; + + + +: 100% activity. Hazy margin, SM: Sharp margin, numbers refer to zone of inhibition (mm) [b]Toxicity of sample to murine leukaemia cell lines (ATCC CCL 46 P388D1) in ng/mL at 0.075 μg/disk. P388: Concentration of the sample required to inhibit cell growth to 50% of a solvent control. [c]Toxicity of sample to murine leukaemia cells (ATCC CCL 46 P388D1) in ng/mL at 7.5 μg/disk. [d]Cytotoxicity in antiviral assays. @ 0.5 mg/mL, 15 μg/disk; Zone of cytotoxic activity: + + + +: 100% activity. [d]Cytotoxicity in antiviral assays. @ 0.25 mg/mL, 15 μg/disk; Zone of cytotoxic activity: + + + +: 100% activity and + + +: 75% activity. [e]Width of zone of inhibition in mm; 15 μg/disk; -: not detected, 0: not determined. Chloramphenicol: 30 mcg/disk, Gentamycin: 30 mcg/disk, Nystatin: 100 unit/disk. HM: Hazy margin, SM: Sharp margin, numbers refer to zone of inhibition (mm)

Table 2. List of microorganisms used for antimicrobial susceptibility test.

Microorganism	Strain
Gram-positive bacterium	
Bacillus subtilis	ATCC 19659
Gram-negative bacteria	
Escherichia coli	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Fungi	
Cladosporium resinae	ATCC 52833
Candida albicans	ATCC 14053
Trichophyton mentagrophytes	ATCC 28185

In conclusion, (-)-*ent*-arbusculin B (**1**) has isolated from the whole plant of *H. paucistipula*, and its structure has determined by spectroscopic analysis. This sequiterpene lactone (**1**) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (2 mm inhibition zone at 15 μg/disk), cytotoxic to murine leukaemia cell lines

ATCC CCL 46 P388D1, (IC_{50} 1,125 ng/mL at 7.5 μg/disk) and BSC monkey kidney cell lines (100% of well at 15 μg/disk).

Acknowledgements

We thank Dr. N. B. Perry at the Plant Extracts Research Unit, New Zealand Institute for Crop & Food Research Ltd, Department of Chemistry, University of Otago in New Zealand. This work was supported by Wonkwang University in 2005.

References

1. Allison, K.W., Child, J. The Liverworts of New Zealand: University of Otago Press, Dunedin, 1975.
2. Asakawa, Y. In progress in the Chemistry of Organic Natural Products: Herz, W., Kirby, G.W., Moore, R.E., Steglich, W., Tamm, C., Eds. Springer Vienma, Vol. 65, 1995.
3. Asakawa, Y., Matsuda, R., Cheminat, A. Bibenzyl derivatives form Frullania species. Phytochemistry, 26, 1117-1122, 1987.
4. Asakawa, Y., Muller, J.C., Ourisson, G., Foussereau, J., Ducombs, G. New sesquiterpene lactones of *Frullania* (Hepaticae). Isolation, structures, allergenic properties. Bull. Soc. Chim. Fr. (Chim. Mol.), pp 1465-1466, 1976.
5. Lorimer, S.D., Barns, G., Evans, A.C., Foster, L.M., May, B.C.H., Perry, N.B., Tangney, R.S. Phytomed., Cytotoxicity and antimicrobial activity of plants from New Zealand's subantarctic islands. 2, 327-333, 1996.
6. Asakawa, Y., Lin, X., Kondo, K., Fukuyama, Y. Chemosystematics of bryophytes. Part 47. Terpenoids and aromatic compounds from selected east Malaysian liverworts. Phytochemistry, 30, 4019-4024, 1991.
7. Kraut, L., Mues, R., Sim-Sim, M. Sesquiterpene lactones and 3-benzylphthalides from *Frullania muscicola*. Phytochemistry, 37, 1337-1346, 1994.
8. Baek, S.H., Perry, N.B., Weavers, R.T., Tangney, R.S. Geranyl phenyl ethers from the New Zealand liverwort *Trichocolea hatcheri*. J. Nat. Prod. 61, 126-129, 1998.
9. Baek, S.H., Lim, J.A., Kwag, J.S., Lee, H.O., Chun, H.J., Lee, J.H., Perry, N.B. Screening for biological activity of crude extract and fractions from *Brachyglottis monroi*. Kor. J. Orien. Physiol. & Pathol. 17(3):826-828, 2003.