Antifungal Activity of (-)-*ent*-Costunolide on the Dermatophytic Fungus *Trichophyton mentagrophytes*

Myung Ju Kim, Seung Hwa Baek[1]*

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

Bioactivity directed isolation has led to the isolation of (-)-*ent*-costunolide (1) as the major active compound. This compound (1) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (4 mm inhibition zone at 15 μg/disc).

Key words : (-)-*ent*-costunolide (1), *Trichophyton mentagrophytes*, Antifungal activity

Introduction

Liverworts have been a rich source of sesquiterpenes, including several new skeletal types.[1,2] When the same compounds have been isolated from liverworts and vascular plants, they are often enantiomeric.[2,3] 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*[4,5] and (+)-arbusculin B from *F. serratta* and *F. muscicola*.[6,7] (+)-Costunolide (3) is known from vascular plants8-10 and from liverworts.8) (-)-*ent*-Arbusculin B (2) has isolated from the whole plant of *Hepatostolonophora* paucistipula (Rodw.) J.J. Engel (family Geocalycaceae), and its structure has determined by spectroscopic analysis. This sequiterpene lactone (2) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (2 mm inhibition zone at 15 μg/disc). In this study, the antiviral and antimicrobial activities of (-)-*ent*-costunolide (1) from *Hepatostolonophora 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 μ m silica gel, Allth) as adsorbent. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F254 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°C, 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 was identified by D. Glenny, Landcare Research, and a voucher specimen, OTA 046764, has been kept in the Otago University herbarium.

3. Isolation of (-)-*ent*-costunolide (1)

Air-dried *H. paucistipula* (76.3 g) was ground and macrated in redistilled ethanol (1,000 mL) in a Waring Blender,

* To whom correspondence should be addressed at : Seung Hwa Baek, Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea
· E-mail : shbaek@wonkwang.ac.kr, · Tel : 063-850-6225
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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 C18-bonded silica column (10 g), developed with H_2O, 3 : 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 CH3CN (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% ethyl acetate : hexane (2 x 4 mL) and 10% ethyl acetate : hexane (5 x 4 mL) were combined and yielded white crystals (44.0 mg, P 388 IC_{50} 302 ng/mL). This material (44.0 mg) was fractionated on a silica gel column (500.0 mg), developed with 100% hexane (4 x 2 mL), 5% (4 x 2 mL), 10% (5 x 2 mL), 15% (5 x 2 mL), 20% (5 x 2 mL), 25% (6 x 2 mL), 30% (6 x 2 mL), mixture of ethyl acetate : hexane. Fractions eluted with 5% ethyl acetate : hexane (4 x 2 mL) and 10% ethyl acetate : hexane (2 x 2 mL) were combined and yielded white crystals (37.1 mg, P 388 IC_{50} 945 ng/mL).

The third P 388-active fraction from this column was subjected to preparative Si gel TLC (1 : 9 ethyl acetate : hexane) to give (-)-*ent*-costunolide (1, 14.4 mg, P 388 IC_{50} 687 ng/mL): courless gum; $[\alpha]^{22}$ -127^0, $[\alpha]^{25}$577 nm -175^0, $[\alpha]^{25}$546 nm -233^0, $[\alpha]^{25}$435 nm -621^0, $[\alpha]^{25}$405 nm -748^0, (c, 0.075, $CHCl_3$); lit. [α]D -35^0, ($CHCl_3$); silica TLC R_F 0.20 (1 : 9 - ethyl acetate : hexane); UVλmax (MeOH) nm (log ε) 224 (4.00); $IR_{\nu max}$ (film), 2921, 2856, 1763, 1665, 1442, 1382, 1289, 1246, 1137, 968, 755 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 6.27 (1H, d, J=3.6 Hz, 13'-H), 5.53 (1H, d=3.3 Hz, 13-H), 4.86 (1H, brdd, J=6.6, 10.8 Hz, 1-H), 4.75 (1H, brd, J=10.2 Hz, 5-H), 4.58 (1H, dd, J=8.7, 9.6 Hz, 6-H), 2.58 (1H, m, 7-H), 2.46 (1H, brdd, J=3.9, 13.5 Hz, 3'-H), 2.0 - 2.4 (6H, m, 2'-H, 3-H, 8-H, 8'-H, 9-H, 9'-H), 1.71 (3H, s, CH_3), 1.67 (1H, dd, J=2.7, 5.1 Hz, 2-H), 1.43 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$): δ 170.52 (C-12), 141.52 (C-11), 140.15 (C-4), 137.00 (C-10), 127.33 (C-5), 127.12 (C-1), 119.70 (C-13), 81.97 (C-6), 50.49 (C-7), 41.07 (C-3), 39.54 (C-9), 28.13 (C-2), 26.27(C-8), 17.42 (C-15), 16.19 (C-14); EI-MS (70 eV): 232.1464 (26%, M^+, $C_{15}H_{22}O_2$ requires 232.1463), 217 (25%, M^+-CH_5), 149 (36%), 136 (15%), 121 (100%); A dose of 15 μg/disk and 7.5 μg/disk showed 100% inhibition of *Herpes simplex* and *Polio*. A dose of 3.75 μg/disk showed 75% inhibition of *Herpes simplex* and *Polio*. 15 μg/disk gave a 4 mm zone of inhibition of *Bacillus subtilis*.

4. Screening for antiviral activity

The compound was applied (30 μL of a 0.5 mg/mL solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder et al. methods.4) The results were observed either cell death (cytotoxicity), inhibition of virus replication, no effect (i.e., all of the cells show viral infection), or a combination of all three. The results were noted as the approximate size of the circular zone, radiating from the extract sample, from 1+ to 4+ representing 25% through to whole well sized zones. The notation used is inhibition/antiviral activity. The type of antiviral effect, indicated by a number after the size of the zone, was also considered important and may give some indication as to the mode of cytotoxic action.

5. Screening for antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested: multiresistant *Bacillus subtilis* (ATCC 19659) *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Cladosporium resinae* (ATCC 52833) and *Candida albicans* (ATCC 14053). The compound was dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 0.5 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 10^4 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. 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).

6. Screening for antifungal activity

Fungal spore suspensions of *Trichophyton mentagraphytes* (ATCC 28185) were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 30 μg sample/disc, and dired at 37℃ for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28℃.

Results and Discussion

1. Isolation and identification of (-)-*ent*-costunolide (1)

We have surveyed the New Zealand liverwort for plant products with potential medicinal and agricultural applications. One of the plant extracts that showed activity in our antiviral (*Herpes simplex*) and antimicrobial (*Trichophyton mentagrophyte* and *Bacillus subtilis*) screens came from the liverwort of Jungermannia species. This liverwort is rich sources of diterpenoids belonging to the pimarane, clerodane and kaurane classess.[2] 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 P388 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.[9] Because of this, the fraction 4 chromatographed on a silica gel column using a ethyl acetate - hexane gradient. The fraction 4-3 with high activity was shown by TLC and ^{1}H-NMR spectrum to consist of one main UV-active compound. The subfraction chromatographed on a silica gel column using a ethyl acetate - hexane gradient. The column subfractions were combined based on visually similar TLC results. These combined subfractions were assayed against P 388 murine leukaemia cell lines (ATCC CCL 46 P388D1) and the activity was found to be spread over four subfractions that were eluted with 100% hexane, 5-10% ethyl acetate / hexane, 10-15% ethyl acetate / hexane, and 20-30% ethyl acetate / hexane. The fraction 4-3-2 with high cytotoxic activity was shown by TLC and ^{1}H-NMR spectrum to consist of one main UV-active compound. Thin-layer chromatography spread cytotoxic activity across ethyl acetate - hexane 10 : 90 (Rf 0.20) band containing (-)-*ent*-costunolide (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 δ5.53 (1H, d, J= 3.3 Hz) and δ6.27 (1H, d, J= 3.6 Hz) and the protons at C-6 in a allylic bond as one-proton double doublet at δ4.75 (brd, J= 10.2 Hz) and δ4.86 (brdd, J=6.6, 10.8 Hz) together with two methyl groups at δ1.43 and 1.71.[7]

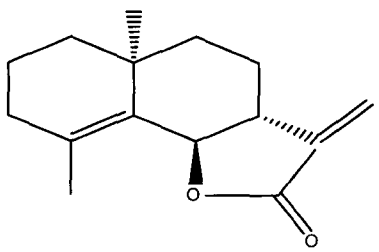

Fig. 1. The structure of (-)-*ent*-costunolide (1)

(-)-*ent*-Costunolide (1) from *H. paucistipula* has known the major sesquiterpene lactone which had ^{1}H and ^{13}C-NMR data matching those reported for (+)-costunolide (2).[8,10] However, the optical rotation ([α]$_D$-127^0) was opposite to the literature values for 2 (+117^0, +129^0).[8,11] The minor sesquiterpene lactone was identified as (-)-*ent*-arbusculin B (3) since it showed the same ^{1}H and ^{13}C-NMR data as those reported for (+)-arbusculin B (4),[8,12] but had a negative optical rotation. We could only find two previous reports of the isolation of 3, also known as (-)-γ-cyclocostunolide, from two *Frullania* species of liverwort.[4,5] It is not surprising that germacranolide 1 and eudesmanolide 3, with the same rare 7α stereochemistry should co-occur, since eudesmanes are thought to be biosynthesised via germacrane intermediates.[13]

2. Antimicrobial activity of (-)-ent-costunolide (1).

As indicated in Table I, the compound (1) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (4 mm inhibition zone at 15 μg/disc). The activities are expressed by the diameter of the developed inhibition zones and compared with those of the widely antibious chloramphenicol, gentamycin and nystatin (Tables 1, 2).[14]

Table 1. Antimicrobial activity of (-)-*ent*-costunolide (1) from *H. paucistipula*

Tested sample	Antimicrobial activitya					
	B. subtilis	*C. albicans*	*T. ment.*	*E. coli*	*C. resinae*	*P. aerug*
1	-	-	SM 4	-	-	-
Chloramphenicol	SM 13	0	0	0	0	0
Nystatin	0	HM 10	SM 6	0	HM 10	0
Gentamycin	0	0	0	SM 9	0	SM 11

[a]Width of zone of inhibition in mm; 15 μg/disc; -: not detected, 0: not determined. Chloramphenicol: 30 mcg/disc, Gentamycin: 30 mcg/disc, Nystatin: 100 unit/disc. HM: Hazy margin, SM: Sharp margin, numbers refer to zone of inhibition (mm)

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

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*-costunolide (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, (4 mm inhibition zone at 15 μg/disc).

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