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ORIGINAL PAPER

Cytotoxic, radical scavenging and antimicrobial activities of sesquiterpenoids from the Tahitian liverwort *Mastigophora diclados* (Brid.) Nees (Mastigophoraceae)

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Abstract A drimane, (+)-drimenol (**1**), five known herbertanes, (−)-α-herbertenol (**2**), (−)-herbertenediol (**3**), mastigophorene A (**4**), (−)-mastigophorene C (**5**) and (−)-mastigophorene D (**6**), a pimarane, (−)-*ent*-pimara-8(14),15-dien-19-oic acid (**7**), and two eudesmanolides, (−)-diplophyllolide A (**8**) and (−)-diplophyllin (**9**) were isolated from the Tahitian *Mastigophora diclados* (Brid.) Nees. Herbertane sesquiterpenes (**2**, **3**, **5** and **6**) showed cytotoxicity against HL-60 and KB cell lines, radical scavenging activity and antimicrobial activity against *Bacillus subtilis*. (−)-Diplophyllolide A (**8**) also exhibited cytotoxicity against HL-60 and KB cell lines.

Keywords Liverwort · *Mastigophora diclados* · Sesquiterpenoid · Cytotoxicity · Radical scavenging · Antimicrobial activity

Introduction

The liverwort *Mastigophora diclados* (Brid.) Nees has been classified into the Mastigophoraceae family [1] and is known to contain monomers and dimers of herbertane sesquiterpenoids, macrocyclic bisbibenzyls, *ent*-trachylobane and *ent*-pimarane diterpenoids [2–9]. These components have also been found in *Herbertus* species [10–16], thus *Mastigophora* and *Herbertus* were concluded to be closely related chemically [4]. Herbertane sesquiterpenoids are chemical markers of *Mastigophora diclados* and it has been reported to possess interesting biological activities. The dimeric herbertanes mastigophorene A (**4**), B and D (**6**) have been found to exhibit neurotrophic activity [6], while α-herbertenol (**2**), β-herbertenol, herbertenediol (**3**), herbertenal, 1,2-diacetoxyherbertene (**10**) prepared from **3**, mastigophorene C (**5**) and mastigophorene D (**6**) inhibited NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells [17]. (−)-α-Herbertenol (**2**), (−)-β-herbertenol, (−)-α-formylherbertenol and (−)-β-bromoherbertenol showed antifungal activity against plant pathogenic fungi *Botrytis cinerea* and *Rhizoctonia solani* [16]. α-Herbertenol (**2**), β-herbertenols, α-formylherberternol and mastigophorene C (**5**) showed antimicrobial activity against bacterium *Staphylococcus aureus* [7].

During a search for biologically active metabolites of liverworts, we found that Et_2O and MeOH crude extracts of the Tahitian *Mastigophora diclados* exhibited cytotoxicity against HL-60 and KB cell lines, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and antimicrobial activity against *Staphylococcus aureus* NBRC 15035 and *Bacillus subtilis* NBRC 3134 as shown in Table 1. Recently, we analysed the ether crude extract of the Tahitian *M. diclados* using GC-MS and reported the presence of herbertene (30.4%), α-herbertenol (**2**) (15.4%), β-herbertenol (1.4%) and herbertenediol (**3**) (21.9%) [18]. Here we report the isolation, structural elucidation and derivatization of chemical components of the Tahitian *M. diclados* as well as results of their cytotoxic, radical scavenging and antimicrobial activities. Structures were established by a combination of extensive NMR, IR and mass spectrometry. Cytotoxic, radical scavenging and

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Table 1 Cytotoxic, radical scavenging and antimicrobial activities of sesquiterpenoids from the Tahitian *Mastigophora diclados*

Compounds	Cytotoxicity IC_{50} (μg/ml)		Radical scavenging IC_{50} (μg/ml)	Antimicrobial MIC (μg/ml)
	HL-60	KB		
Et_2O crude extract	2.4 ± 0.5	14.6 ± 0.8	21.4 ± 0.7	*B. subtilis* (16) *S. aureus* (16)
MeOH crude extract	13.1 ± 2.8	32.6 ± 1.5	20.8 ± 0.7	*B. subtilis* (64) *S. aureus* (64)
(+)-Drimenol (**1**)	–	–	–	–
(−)-α-Herbertenol (**2**)	12.8 ± 0.7	12.5 ± 0.6	–	*B. subtilis* (8)
(−)-Herbertenediol (**3**)	1.4 ± 0.1	11.8 ± 1.4	1.9 ± 0.6	*B. subtilis* (8)
Mastigophorene A (**4**)	–	–	–	–
(−)-Mastigophorene C (**5**)	2.4 ± 0.1	14.8 ± 1.1	2.7 ± 0.8	*B. subtilis* (8)
(−)-Mastigophorene D (**6**)	2.5 ± 0.4	14.2 ± 1.0	2.0 ± 0.1	*B. subtilis* (2)
(−)-*ent*-Pimara-8(14),15-dien-19-oic acid (**7**)	>100	>100	>100	>100
(−)-Diplohyllolide A (**8**)	1.4 ± 0.2	3.3 ± 1.2	>100	>100
(−)-Diplophyllin (**9**)	–	–	–	–
(−)-1,2-Diacetoxyherbertene (**10**)	2.6 ± 0.0	15.0 ±0.5	>100	*B. subtilis* (16)
(−)-1-Hydroxy-2-methoxyherbertene (**11**)	11.0 ± 0.9	14.9 ± 1.2	23.2 ± 0.7	*B. subtilis* (32)
Mytomicin C	0.016 ± 0.001	0.011 ± 0.001	–	–
Vitamin C	–	–	2.6 ± 1.0	–
Quercetin	–	–	1.9 ± 0.6	–
Ampicillin	–	–	–	< 2

–, Not tested because the isolated compounds were obtained in low yields (1–3 mg)

antimicrobial activities were evaluated by using WST-8 colorimetric, DPPH and dilution assays, respectively.

Materials and methods

General experimental procedures

Optical rotations were measured on JASCO P 1030 polarimeter. IR spectra were recorded on a Shimadzu FTIR-8400S infrared spectrometer. The ^{1}H- and ^{13}C-NMR were measured on Varian unity-600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) and Varian Unity-500 MHz (^{1}H, 500 MHz; ^{13}C, 125 MHz) instruments. Chemical shift values were expressed in δ (ppm) downfield from trimethylsilane (TMS) as an internal standard, and in δ 77.03 (ppm) from $CDCl_3$ as a standard (^{13}C-NMR). Mass spectra were obtained on JEOL JMS 700 instrument. Column chromatography was performed on silica gel 60 (0.063–0.200 mm) (Merck) and Sephadex LH 20 (Amersham Pharmacia Biotech, eluant MeOH–CH_2Cl_2 1:1). HPLC was carried out on Cosmosil 5SL-II (10 × 250 mm).

Plant material

Mastigophora diclados (Brid.) Nees was collected at Mount Marau, 1400-m altitude (Tahiti) October 2007. The species was identified by Dr. A. Pham from the Herbarium of the Université de Polynésie Française (UPF). The voucher specimen was deposited in UPF and Tokushima Bunri University.

Extraction and isolation

The air-dried and ground *Mastigophora diclados* (106.5 g) was extracted with Et_2O and then MeOH. Filtration and removal of solvent in vacuo gave the Et_2O (3.5 g) and MeOH (4.0 g) crude extracts, respectively. Fractionation of Et_2O extract (3.5 g) upon silica gel (hexane–EtOAc gradient) gave eleven fractions. Fraction 5 (2.2 g) was further purified (silica gel, Sephadex LH-20, preparative HPLC, and hexane–EtOAc) to give (−)-α-herbertenol (**2**, 3 mg) $[\alpha]_D$ −62.9 (*c* 1.0, $CHCl_3$) [lit. −55.0] [14], (−)-herbertenediol (**3**, 49 mg) $[\alpha]_D$ −33.5 (*c* 1.0, $CHCl_3$) [lit. −46.5] [15], mastigophorene A (**4**, 1 mg), (−)-*ent*-pimara-8(14),15-dien-19-oic acid (**7**, 15 mg) $[\alpha]_D$ −155.3 (*c* 1.0, $CHCl_3$) [lit. −120.6] [19], (−)-diplophyllolide A (**8**, 5 mg) $[\alpha]_D$ −110.4 (*c* 1.0, $CHCl_3$) [lit. −132.0] [20], (−)-diplophyllin (**9**, 3 mg) $[\alpha]_D$ −79.4 (*c* 1.0, $CHCl_3$) [lit. −108.0] [21] and (+)-drimenol (**1**, 2 mg) $[\alpha]_D$ +54.4 (*c* 1.0, $CHCl_3$) [lit. +20.0] [22]. Fraction 6 (165 mg) was purified by using a combination of column chromatography on silica gel (hexane–EtOAc gradient), Sephadex LH-20 and preparative HPLC (hexane–EtOAc 4:1) to give (−)-mastigophorene C (**5**, 5 mg) $[\alpha]_D$ −60.7 (*c* 1.0, $CHCl_3$) [lit.

−46.7]) [5], while purification of fraction 7 (231 mg) using HPLC (hexane–EtOAc 3:2) gave (−)-mastigophorene D (**6**, 17 mg) $[\alpha]_D$ −68.2 (*c* 1.0, $CHCl_3$), [lit. −46.1] [5]. Fractionation of MeOH extract (4.0 g) upon silica gel (hexane–EtOAc gradient) gave ten fractions. Fraction 7 (100 mg) was purified upon silica gel, preparative HPLC (hexane–EtOAc 3:2) and Sephadex LH-20 to give (−)-herbertenediol (**3**, 54 mg) and (−)-diplophyllolide A (**8**, 2 mg). Fraction 8 (136 mg) was further purified (silica gel, Sephadex LH-20, preparative HPLC, hexane–EtOAc gradient and hexane–EtOAc 3:2) to give (−)-mastigophorene D (**6**, 7 mg). The ^{1}H-NMR and ^{13}C-NMR spectra of compounds **1**–**10** were identical to authentic samples.

*Acetylation of herbertenediol (**3**)*

(−)-Herbertenediol (**3**, 7 mg) was dissolved in a mixture of Ac_2O (1 ml) and pyridine (2 ml), and then the solution was stored at room temperature overnight. The solution was evaporated by using a freeze dryer and then extracted with EtOAc. Purification upon silica gel (hexane–EtOAc 4:1) gave (−)-1,2-diacetoxyherbertene (**10**, 9 mg) $[\alpha]_D$ −51.2 (*c* 1.0, $CHCl_3$) [lit. −22.6] [7, 16].

*Methylation of herbertenediol (**3**)*

(−)-Herbertenediol (**3**, 12 mg) was dissolved in acetone (1 ml), and then K_2CO_3 (7 mg) and MeI (100 µl) were added. The solution was refluxed for 1 h. Work-up as usual gave (−)-1-hydroxy-2-methoxyherbertene (**11**, 1.6 mg) as needle crystals, m.p. 36–37°C; $[\alpha]_D$ −65.0 (*c* 1.0, $CHCl_3$); HR-EIMS: 248.1780, calcd. 248.1776 for $C_{16}H_{24}O_2$; FTIR ($CHCl_3$) ν_{max} 3525, 2956, 2871, 1595, 1487, 1460, 1417, 1371, 1292, 1230, 1166, 1145, 1058 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz): 0.74 (3H, s, H-15), 1.17 (3H, s, H-14), 1.41 (3H, s, H-13), 1.53 (1H, m, H-10a), 1.64 (1H, s, H-10b), 1.75 (1H, m, H-8a), 1.75 (2H, m, H-9), 2.28 (3H, s, H-12), 2.60 (1H, m, H-8b), 3.86 (3H, s, OCH_3), 5.84 (1H, s, OH), 6.57 (1H, s, H-3), 6.72 (1H, s, H-5); ^{13}C-NMR ($CDCl_3$, 125 MHz): 20.5 (C-9), 21.5 (C-12), 22.7 (C-13), 25.6 (C-14), 27.1 (C-15), 39.1 (C-8), 41.2 (C-10), 44.8 (C-11), 51.2 (C-7), 56.1 (OCH_3), 109.0 (C-3), 121.8 (C-5), 127.0 (C-4), 132.5 (C-6), 142.5 (C-1), 146.4 (C-2). EI-MS *m/z* (rel. int.): 248 [M^+] (88), 233 (4), 205 (5), 192 (12), 178 (64), 166 (100), 145 (13), 91 (14), 69 (7), 41 (9).

Biological assays

Cytotoxicity assay

Cell lines used in this study were HL-60 cells (human promyelocytic leukaemia) and KB cells (human pharyngeal squamous carcinoma), both purchased from DS Pharma Biomedical, Osaka, Japan. HL-60 cells were non-adherent cells and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. KB cells were grown as monolayer in MEM supplemented with 10% FBS and 1% non-essential amino acid at 37°C in a humidified atmosphere of 5% CO_2. HL-60 or KB cells were seeded in 96-well plates at a density 5×10^3 cells/well (100 µl/well), and the cultures were incubated for 3 h (HL-60 cells) or 24 h (KB cells). Then, 100-µl aliquots of medium containing test compounds were added, and the cells were incubated for 72 h (HL-60 cells) or 48 h (KB cells). Cytotoxicity assays were performed by adding 20 µl of WST-8 solution to the cultures and incubating for 1–2 h at 37°C; after incubation, the optical densities at 450 nm were measured by using a microplate reader (Molecular Device, Tokyo, Japan).

DPPH free radical scavenging assay

The pure compounds were spotted on TLC plates and then the plate developed and dried. The TLC plate was sprayed with 0.2% of 1,1-diphenyl-2-picrylhydrazyl (DPPH) methanolic solution and stored in the dark. The spots on TLC plates were observed under ordinary light after 30 min [23]. This method was used to observe whether these compounds were active or not. In order to determine the IC_{50} values of the active compounds, the scavenging activity of pure compounds on 1,1-diphenyl-2-picrylhydrazyl (DPPH, Nacalai Tesque, Kyoto) was measured according to a previous method [24]. Various concentrations of samples (0.1 ml) were mixed with 0.1 ml of 50 µM DPPH solution in 96-well microtiter plates. After 30 min of incubation at room temperature the optical densities at 517 nm were measured by using a microplate reader (Molecular Device, Tokyo, Japan). The percentage of scavenging of DPPH radical on samples was calculated by using the following equation:

$$\%\,\text{inhibition} = 1 - [\text{OD(DPPH + sample)/OD(DPPH)}] \times 100\%$$

Antimicrobial assay

The following microorganisms were used for testing antimicrobial activity: *Bacillus subtilis* NBRC 3134 and *Klebsiella pneunomiae* NBRC 3512. Bacteria strains were tested by using microdilution assays and minimum inhibitory concentration (MIC) values were determined. Bacterial strains were inoculated on YP agar plates (1% polypeptone, 0.2% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$ and 6% agar) and were incubated at 37°C (*B. subtilis*) and 30°C (*K. pneunomiae*) for 12 h. The stock solution of crude extracts was

prepared in 10 mg/ml in DMSO and further diluted to varying concentration in 96-well plates which have contained incubated microbial strains. Each plate was further incubated in 37°C for overnight and ampicillin in varying concentration was used as reference drug.

Result and discussions

Mastigophora diclados was extracted with Et_2O and MeOH and then fractionated by using a combination of column chromatography on silica gel, Sephadex LH-20 and preparative HPLC to give (+)-drimenol (**1**) [22], (−)-α-herbertenol (**2**) [14], (−)-herbertenediol (**3**) [6], mastigophorene A (**4**), (−)-mastigophorene C (**5**), (−)-mastigophorene D (**6**) [5, 6], (−)-*ent*-pimara-8(14),15-dien-19-oic acid (**7**) [25], (−)-diplophyllolide A (**8**) [26] and (−)-diplophyllin (**9**) [21] (Fig. 1). Acetylation and methylation of (−)-herbertenediol (**3**) were carried out in order to compare the biological activity of the resulting derivatives with the natural herbertanes.

This is the first reported isolation of (+)-drimenol (**1**) from a natural source, although it has been reported as a synthetic compound [22]. The IR, MS and NMR spectra of (+)-drimenol (**1**) were identical to those (−)-drimenol [27], except for the sign of optical rotation. (−)-Drimenol has been found in higher plants [28, 29] and the different liverworts, *Diplophyllum* (Scapaniaceae) [21], *Bazzania* (Lepidoziaceae) [27], *Targionia* (Targioniaceae) [30], *Frullania* (Frullaniaceae) [31] and *Porella* (Porellaceae) [29].

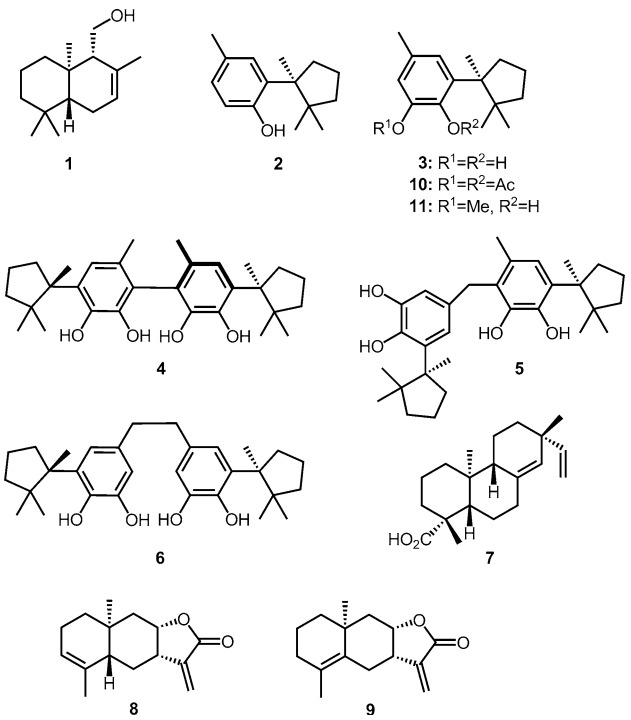

Fig. 1 Isolated sesqui- and diterpenoids from the Tahitian *Mastigophora diclados*

Herbertane sesquiterpenoids (**2**–**6**) are chemical markers of *M. diclados* and they have been found as major components in the Tahitian *M. diclados*. Together with (+)-drimenol and (−)-herbertanes, two (−)-eudesmanolides, (−)-diplophyllolide A (**8**) and (−)-diplophyllin (**9**), were also isolated from this liverwort. Previously, **8** and **9** have been isolated from the liverworts *Diplophyllum* (Scapaniaceae) [21], *Plagiochila* (Plagiochilaceae) [32], *Clasmatocolea* (Lophocoleaceae) [33], *Chiloscyphus* (Lophocoleaceae) [33] and *Tritomaria* (Scapaniaceae) [34].

The isolated herbertanes (**2**, **3**, **5** and **6**) exhibited cytotoxicity against HL-60 and KB cell lines, DPPH radical scavenging activity and antimicrobial activity against *Bacillus subtilis* as shown in Table 1. Since these compounds possess hydroxyl groups, the derivatization of the major component (−)-herbertenediol (**3**) was carried out to investigate whether the biological activities were caused by the presence of hydroxyl groups or not. The herbertane derivatives (−)-1,2-diacetoxyherbertene (**10**) and (−)-1-hydroxy-2-methoxyherbertene (**11**) were tested for their cytotoxic, radical scavenging and antimicrobial activities. When tested against KB cells, there was no significant decrease in cytotoxicity of **10** and **11** (relative to **3**), suggesting that the hydroxyl group does not play an important role in providing cytotoxicity against KB cells. When they were tested against HL-60 cells, 1,2-diacetoxyherbertene (**10**) did not show any significant decrease in activity; however, natural α-herbertenol (**2**) and 1-hydroxy-2-methoxyherbertene (**11**) had reduced cytotoxic activity against HL-60 cells as shown in Table 1. There is a possibility that the position of substitution on the benzene ring will influence the cytotoxicity against HL-60 cells; however, because of the lack of related compounds available for testing, we cannot conclude which structural features influenced the decrease in cytotoxicity of both compounds (**2** and **11**) against HL-60 cells. (−)-Diplophyllolide A (**8**) exhibited strong inhibitory activity against HL-60 and KB cell lines with IC_{50} values of 1.4 and 3.3 μg/ml, respectively. (−)-Diplophyllolide A (**8**) was reported to be toxic against P388 cells [33], while (−)-diplophyllin (**9**) was toxic against KB cells [21]. Ohta et al. [21] concluded that the cytotoxicity of eudesmanolide is due to the presence of the methylene lactone function. Among all the isolated sesquiterpenes and derivatives tested, (−)-herbertenediol (**3**) and (−)-diplophyllolide A (**8**) were the most inhibitory active sesquiterpenes against the HL-60 cell line; on the other hand, only (−)-diplophyllolide A (**8**) showed strong cytotoxic activity against the KB cell line.

Since the herbertanes (**2**, **3**, **5** and **6**) possess hydroxyl groups, there was no doubt that the radical scavenging

activity of these herbertanes is due to the presence of this functional group. This phenomenon was supported by the negative result for (−)-1,2-diacetoxyherbertene (**10**) and reduced activity of (−)-1-hydroxy-2-methoxyherbertene (**11**) (relative to **3**, **5** and **6**) in the DPPH free radical scavenging assay. Radical scavenging activities of (−)-herbertenediol (**3**) and (−)-mastigophorene D (**6**) were higher than vitamin C and similar to quercetin.

The herbertane sesquiterpenoids (**2**, **3**, **5** and **6**) were also tested for antimicrobial activity against *Bacillus subtilis* and *Klebsiella pneunomiae*. These herbertanes were active against *B. subtilis* as shown in Table 1, but only herbertenediol (**3**) showed weak activity against *K. pneunomiae* (MIC at 100 μg/ml). Among all of the herbertanes, the dimeric herbertane (−)-mastigophorene D (**6**) is the most active sesquiterpene against *B. subtilis* (MIC at 2 μg/ml). Derivatization of herbertenediol (**3**) caused decreased antimicrobial activity against *B. subtilis*, relative to (−)-1,2-diacetoxyherbertene (**10**) and (−)-1-hydroxy-2-methoxyherbertene (**11**) (Table 1).

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