Chemical Constituents of Malagasy Liverworts, Part II[1]: Mastigophoric Acid Methyl Ester of Biogenetic Interest from *Mastigophora diclados* (Lepicoleaceae Subf. Mastigophoroideae)

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In the course of our chemotaxonomic study of the liverworts growing in Madagascar, mastigophoric acid methyl ester, along with eleven known compounds were isolated from *Mastigophora diclados*. Isolated metabolites showed that the Malagasy *Mastigophora* is more related to the samples from Borneo and Japan than to the Taiwanese or Malaysian ones. The biosynthesis of the herbertane type sesquiterpenoids from *Mastigophora diclados* is suggested to be similar to those found in the genus *Herbertus*. The herbertane-type sesquiterpenoids were screened for *Staphylococcus aureus* strain inhibition.

Key words liverwort; Lepicoleaceae subf. Mastigophoroideae; *Mastigophora diclados*; chemosystematic; biogenesis; antibacterial activity

The liverwort *Mastigophora diclados* (BRID.) NEES has received much attention in the last decade since the isolation of the dimeric neurotropic compounds: Mastigophorene A—D.[2,3] *M. diclados* is bryologically classified into the family Lepicoleaceae, subfamily Mastigophoroideae.[4] It was however obvious that this genus is chemically more close to *Herbertus* (Herbertaceae) than the other two genera *Ptilidium* (Ptilidiaceae) and *Lepicolea* (Lepicoleaceae).[5] Investigation of the terpenoids and lipophilic aromatic constituents of bryophytes, which have very small gametophytes, can contribute to their classification. Therefore, Asakawa has confirmed the relationship between the genera *Mastigophora* and *Herbertus*, both having herbertane-type sesquiterpenoids as chemical markers.[6] Geographical differences may influence the chemical constituents of the plants. The phytochemical investigation of *Mastigophora diclados* from Taiwan, Borneo, West Malaysia and Japan has shown the presence of herbertene (**1**), α- and β-herbertenols (**2**, **3**), herbertene 2,3-diol (**4**), (−)-sandaracopimaric acid, 12-chloroisoplagiochin D (**5**) and 2,12-dichloroisoplagiochin (**6**), mastigophorenes A—D (**7**—**10**), *ent*-trachiloban-18-oic acid, its C-4 epimer (**11**, **12**) and *ent*-18-hydroxytrachyloban-19-oic acid (**13**).[2—4,7—11] Investigation of the *Mastigophora diclados* collected from Madagascar led to the isolation of mastigophoric acid methyl ester (**14**) together with 11 known compounds. Variations in the chemical constituents in different localities, the possible biosynthesis route of herbertane sesquiterpenoid metabolites in the genus *Herbertus* and *Mastigophora*, as well as the antibacterial properties of the herbertane type sesquiterpenoid are discussed.

Results and Discussion

GC-MS examination of the dried *M. diclados* ether extract (green oil) showed the presence of herbertene (**1**, 17%), 1-octen-3-ol (2.13%), and 1-octen-3-yl acetate (1.87%). The remaining extract was chromatographed on silica gel to afford eighteen fractions. Each fraction was purified with size exclusion chromatography by Sephadex LH-20, silica gel and preparative HPLC or TLC to yield α- and β-herbertenols (**2**, **3**),[12,13] herbertene 1,2-diol (**4**), sandaracopimaric acid and *ent*-pimara-8(14),15-dien-19-oic acid,[14,15] mastigophorene C and D (**9**, **10**),[2,3] riccardin C (**15**),[16] α-formylherbertenol (**16**),[12] stigmasterol, mastigophoric acid methyl ester (**14**) and a mixture of two cycloartenyl esters (**17**). The known compounds were identified by comparison of their spectral data with those of authentic samples and those reported in the literature.

Compound **14** was obtained as an amorphous powder with the molecular formula of $C_{16}H_{22}O_3$ as determined by high-resolution FAB-MS. Its IR and UV spectra showed absorption bands for an ester (1693 cm^{-1}) and a benzene ring (217, 263 nm). The ^{1}H-NMR (see Experimental) spectral data showed the presence of a trisubstituted aromatic ring with ABX coupling [δ_H 8.06 (d, J=1.9 Hz), 7.77 (dd, J=8.2, 1.9 Hz), 6.70 (d, J=8.2 Hz)], four methyls including three quaternary methyl groups (δ_H 1.20, 1.41, 0.74), and one methyl ester (δ_H 3.87). The ^{13}C-NMR spectral data (see Experimental) displayed 16 carbon signals including one esterified carboxyl group (δ_C 167.2), one methyl ester (δ_C 51.8), aromatic carbons [δ_C 158.7 (s), 133.3 (s), 131.6 (d), 129.0 (d), 122.1 (s), 116.7 (d)], and three methylene carbons (δ_C 20.2, 39.5, 41.2). The above data were very similar to those of compound **2**,[12] except for the absence of the downfield methyl signal at δ 2.25 of **2** in the ^{1}H-NMR of **14**, suggesting that the ester group was located at C-12. This fact was confirmed by the downfield shifts (while comparing to those of **2**) of the aromatic proton signals. In order to confirm the location of the functionalities, extensive two dimensional NMR experiments were carried out. Long-range correlations were observed in H-3/C-2, C-3, C-4 and C-12, H-5/C-1, C-4, C-6 and C-12, while the partial structure –CH_2–CH_2–CH_2– of the five member ring could be drawn from the ^{1}H–^{1}H COSY spectrum. Therefore, mastigophoric acid methyl ester (**14**) was determined to be 1-hydroxyherbertene 12-oic acid methyl ester as shown in Chart 1. Although compound **14** has been obtained from one step in the synthesis of (−) α-formylherbertenol,[17] this is the first report on the isolation of compound **14** from a natural source.

Inspection of the NMR spectral data of compound **17** showed that it is a mixture of cycloartenyl esters. Cycloartenol **17a** and a mixture of eicosapentaenoic acid and 11,14-octadecadienoic acid were obtained on saponification of **17**

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with 1 M NaOH in MeOH. The identity of the fatty acids was confirmed by GC-MS of the methyl ester derivatives. Buchanan and coworkers have isolated a similar mixture of compounds with linoleic, α-linoleic, arachidonic, eicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic, palmitic and isostearic acid esters of cycloartenyl.[18] Recently Toyota *et al.*[19] has isolated a series of fatty acid esters of triterpene and sterol by purification on silica gel impregnated silver nitrate column chromatography.

The herbertenoids (−)-α-herbertenol (**2**), (−)-β-herbertenol (**3**), (−)-α-formylherbertenol (**16**), and (−)-β-bromoherbertenol have displayed strong growth-inhibitory activity of two plant pathogenic fungi (*Botrytis cinerea* and *Rhizoctonia solani*).[12] In order to obtain more antimicrobial data for herbertenoids, compounds **2**—**4**, **9**, **14**, **16** and (−)-1,2-dihydroxyherberten-12-al (**18**) were tested against *Staphyloccocus aureus* strain by agar diffusion. Although the herbertenoids tested showed weaker activities than the standard antibiotics used as control [chloramphenicol (22 mm) and kanamycin (23 mm)], Mastigophorene C (**9**), which is a dimer of herbertene-1,2-diol (**4**), exhibited the strongest inhibition (17 mm) while its monomer displayed significant activity (13 mm). α- and β-herbertenols, and α-formylherbertenol showed almost the same inhibition (15, 16, 16 mm, respectively) against *S. aureus* strain.

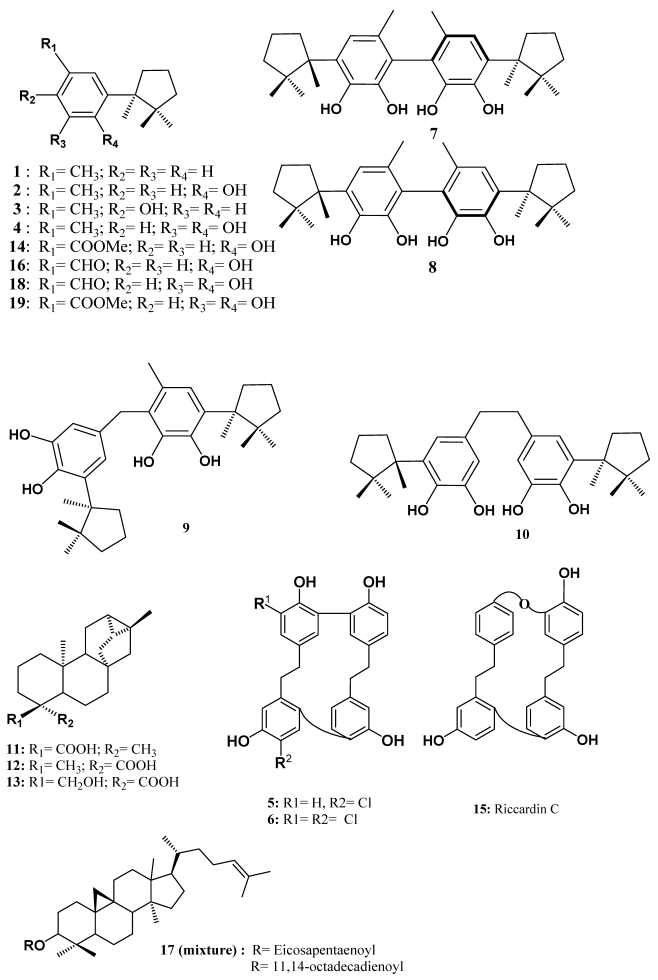

Chart 1. *Mastigophora diclados* Metabolites

Mastigophora diclados is very close to the *Herbertus* species. Possible biogenetic pathways of herbertane-type sesquiterpenoids in *Herbertus sakuraii* have been reported.[20] Very similar pathways could be drawn for *Mastigophora diclados*. Although α-formylherbertenol has been found in *Herbertus*,[4] its acid derivative (**14**) has been isolated only in the genus *Mastigophora*. However, (−)-1,2-dihydroxyherberten-12-al (**18**) and methyl 1,2-dihydroxyherberten-12-oate (**19**) were both isolated from *Herbertus aduncus* but not *Mastigophora diclados*.[17] The difference between the two genus might be in the levels of the oxidation enzymes. Further investigation should be undertaken to identify the enzyme responsible for the oxidation of the metabolites in each plant. It is also interesting to note that riccardin C (**15**) which has a similar structure to 12-chloroisoplagiochin D (previously isolated from the Japanese *M. diclados*),[7] was isolated for the first time from the title plant. The variation in the constituents of *Mastigophora diclados* depending on the geographical location is summarized in Table 1. The *ent*-trachylobane diterpenoids (**11**—**13**) have been detected only in the West Malaysian sample. It could be biogenetically derived from pimarane diterpenoids in the present plant study. Mastigophorenes have only been detected in samples from Borneo and Madagascar while the chlorinated bis-bibenzyls isoplagiochins have been found in Japanese species. At least two chemotypes (mastigophorenes and isoplagiochin) of *M. diclados* has been considered.[7] The isolation of riccardin C, pimarane, and pimarane-derived trachylobane diterpenoids is indicative of three chemotypes in this species: bis-bibenzyls, mastigophorenes and pimarane or pimarane-derived trachylobane diterpenoids. Three chemotypes have been detected from Malagasy *M. diclados*. The differences in the occurrence of chemotypes depending to the geographical location will be the subject of our next investigation.

Experimental

General Procedures Optical rotations were measured on a JASCO DIP-1000 polarimeter with MeOH or $CHCl_3$ as solvent. UV spectra were obtained on a Shimadzu UV-1650PC instrument in MeOH. IR spectra were

Table 1. Chemotype Variations of *Mastigophora diclados*

Species location	Compounds		
	Mastigophorenes	Pimarane/trachylobane	Bis-bibenzyls
Borneo	+	−	−
Japan	−	−	+
Madagascar	+	+	+
Malaysia (West)	−	+	−
Taiwan	−	+	−

+; −: Presence (+) and absence (−) in the plant material.

measured on a JASCO FT/IR-5300 spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Unity 600 NMR spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C), using either $CDCl_3$ or CD_3OD as solvent. Chemical shifts are given relative to tetramethylsilane (TMS, δ 0.00) as internal standard (^{1}H) and δ 77.02 (ppm) from $CDCl_3$ as standards (^{13}C). Mass spectra were recorded on a JEOL JMS AX-500 spectrometer. Column chromatography was carried out on Sephadex LH-20 (Amersham Pharmacia Biotech, $CHCl_3$–MeOH, 1 : 1) and silica gel (Kieselgel 60: 0.040—0.063 mm, Merck). The preparative HPLC experiments was performed using a Cosmosil reverse phase column, a JASCO 880-PU pump, JASCO 875-UV UV detector and ERC-7512 ERMA CR INC RI detector. 20×20 cm RP-18 F_{254S} (Merck) was used for preparative TLC. The temperature programming of GC-MS analysis was performed from 50 °C, then 50—250 °C at 15 °C min^{-1} and finally isothermal at 250 °C. A fused silica column coated with DB-17 (30 m×0.25 mm i.d., film thickness 0.25 mm) using He as carrier gas (1 ml min^{-1}) was used. Mass spectra were measured at 70 eV.

Plant Material *Mastigophora diclados* (Brid.) Nees was collected in Moramanga (Madagascar) in June 2003 and identified by Prof. Dr. Emeritus T. Pocs of the Hungarian Academy of Science. A voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

Extraction and Isolation Dried *M. diclados* (105 g) was mechanically powdered and extracted with ether (1000 ml) at room temperature for 5 d, followed by MeOH for one week. Each extract was filtrated and concentrated *in vacuo* to yield green oils (ether extract: 2.25 g and MeOH extract: 2.64 g). The former extract was subjected to silica gel chromatography using a hexane–EtOAc gradient to divide it into ten fractions. Fraction 1 was applied to a Sephadex LH-20 column to afford **2** (25 mg). Size exclusion chromatography of fraction 2 yielded **3** (5.8 mg), *ent*-pimara-8(14), 15-dien-19-oic acid (11.2 mg) and sandaracopimaric acid (38 mg). Compound **4** (580 mg) was obtained from fraction 3 using a Sephadex LH-20 column and preparative HPLC (70% aqueous CH_3CN). Fraction 4 was divided into three sub-fractions by applying to Sephadex LH-20 chromatography before purifying by RP-18 prep. TLC (80% aqueous CH_3CN) to give mastigophoric acid methyl ester **14** (4.7 mg). Fractions 5, 6, 7 and 8 were purified by Sephadex LH-20 to afford **16** (0.6 mg), mastigophorene C (**9**, 2.2 mg), stigmasterol (25 mg) and **10** (25 mg), respectively. Fraction 10 was divided into 3 sub-fractions and purified by reversed phase RP-18 prep. TLC (90% aqueous CH_3CN) to give **15** (3.8 mg).

The MeOH extract of *M. diclados* was partitioned between H_2O and EtOAc. The organic layer was evaporated *in vacuo* and chromatographed on silica gel using hexane–EtOAc (4 : 1) as eluent to yield **17** (3.6 mg).

Mastigophoric Acid Methyl Ester (**14**): Amorphous powder, $[\alpha]_D^{20}$ −53.6° (c=1.9, MeOH). HR-FAB-MS m/z: 262.1560 $[M]^+$, $C_{16}H_{22}O_3$, requires 262.1569. UV λ_{max} (MeOH) nm (log ε): 217 (2.5), 263 (2.5). IR (KBr) cm^{-1}: 2959, 1693, 1601, 1512, 1420, 1286, 1149, 986. ^{1}H-NMR ($CDCl_3$) δ: 0.74 (3H, s, H-15), 1.20 (3H, s, H-14), 1.41 (3H, s, H-13), 1.56 (1H, m, H-10b), 1.69 (1H, m, H-10a), 1.80 (3H, m, H-9ab, H-8b), 2.63 (1H, m, H-8a), 3.87 (3H, s, OCH_3), 6.70 (1H, d, J=8.2 Hz), 7.77 (1H, dd, J=8.2, 1.9 Hz), 8.06 (1H, d, J=1.9 Hz); ^{13}C-NMR ($CDCl_3$): 20.2 (C-9), 22.6 (C-13), 25.5 (C-14), 26.9 (C-15), 39.5 (C-8), 41.2 (C-10), 44.6 (C-11), 51.0 (C-7), 51.8 (OCH_3), 116.7 (C-2), 122.1 (C-4), 129.0 (C-3), 131.6 (C-5), 133.3 (C-6), 158.7 (C-1) and 167.2 (C-12).

Cycloartenyl Esters (**17**): Oil, $[\alpha]_D^{20}$ +23.2° (c=0.2, $CHCl_3$). EI-MS (rel. int.) m/z: 712 $[M]^+$, 43 (67), 69 (100), 176 (64), 409 (52). IR (KBr) cm^{-1}: 2932, 2668, 1732, 1456, 1376, 1096, 984. ^{1}H-NMR ($CDCl_3$) δ: 0.33 (1H, d, J=4.3 Hz, H-19exo), 0.57 (1H, d, J=4.3 Hz, H-19endo), 0.84 (3H, s), 0.87 (3H, d, J=6.7 Hz), 0.89 (3H, s), 0.95 (6H, s), 1.60 (3H, s), 1.68 (3H, s), 4.58 (1H, m, $W_{1/2}$=13 Hz), 5.10 (1H, m), 5.36 (1H, m); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 18.0, 18.2, 19.2, 20.1, 20.9, 22.6, 24.9, 25.0, 25.1, 25.4, 25.6, 25.7, 25.8, 25.9, 26.5, 26.6, 26.8, 27.2, 28.1, 29.1, 29.3, 29.7, 29.8, 31.5, 31.6, 31.9, 32.8, 34.2, 34.8, 35.5, 35.9, 36.3, 39.4, 45.2, 47.1, 47.8, 48.8, 52.2, 80.3, 80.4 (major), 125.2, 127.5, 127.9, 128.0, 128.2, 128.5, 128.7, 129.0, 130.0, 130.2, 130.5, 130.9, 173.3, 173.6 (major).

Alkaline Hydrolysis of 17 Compound **17** (3.2 mg) was refluxed with 1 M NaOH in MeOH (2 ml) for 2 h. The reaction mixture was poured into H_2O and extracted with EtOAc. The products were purified by silica gel column chromatography (hexane : EtOAc, 4 : 1) to afford **17a** (1.2 mg) and **17b** (0.9 mg). **17a**[21]: ^{1}H-NMR ($CDCl_3$) δ_H: 0.32 (1H, d, J=4.3 Hz, H-19exo), 0.55 (1H, d, J=4.3 Hz, H-19endo), 0.80 (3H, s, CH_3-31), 0.87 (3H, d, J=6.7 Hz, CH_3-21), 0.88 (3H, s, CH_3-32), 0.96 (6H, s), 1.60 (3H, s, CH_3-27) and 1.68 (3H, s, CH_3-26), 1.50 (m, H-8), 3.27 (dd, J=12, 3.9 Hz, H-3), 5.09 (m, H-24); ^{13}C-NMR ($CDCl_3$) δ: 14.0 (C-31), 17.6 (C-26), 18.0 (C-18), 18.2 (C-21), 19.3 (C-32), 20.0 (C-9), 21.1 (C-6), 24.9 (C-23), 25.4 (C-30), 25.7 (C-27), 26.0×2 (C-7, C-10), 26.4 (C-11), 28.1 (C-16), 29.9 (C-19), 30.4 (C-2), 31.9 (C-1), 32.9 (C-12), 35.5 (C-15), 35.9 (C-20), 36.3 (C-22), 40.5 (C-4), 45.2 (C-13), 47.1 (C-5), 48.0 (C-8), 48.8 (C-14), 52.3 (C-17), 78.8 (C-3), 125.2 (C-24), 130.9 (C-25).

Methylation of Compound 17b Compound **17b** (0.9 mg) was dissolved in 1 ml of MeOH/$(CH_3)_3SiCHN_2$ (v/v). The mixture was stirred for 2 h at room temperature. The reaction mixture was evaporated and analyzed by GC/MS to detect the presence of eicosa-5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-pentaenoic acid methyl ester and 11,14-octadecadienoic acid methyl ester at a ratio of 3 : 1.

Antimicrobial Activity Antibacterial assays were carried out by the disc diffusion method. Twenty milliliters of nutrient agar was placed in sterile Petri dishes and inoculated with 18 h L-Broth culture of *Staphylococcus aureus* (IFO 12732). Sterile paper discs (6 mm in diameter) were impregnated with 20 μl (concentration: 1 mg/ml in EtOH) of each compound (**2**—**4**, **9**, **14**, **16**, **18**) and placed in the Petri dishes after evaporation of the solvent under sterile conditions. The plates were incubated for 48 h at 35 °C. The inhibition zone diameter of bacteria around the disc was measured. Chloramphenicol and kanamycin (Showa Yakuhin Kakou; discs, 8 mm diameter, 100 μg/disc) were used as control.

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