Chemical Constituents of Malagasy Liverworts, Part V: Prenyl Bibenzyls and Clerodane Diterpenoids with Nitric Oxide Inhibitory Activity from *Radula appressa* and *Thysananthus spathulistipus*

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3β,4β:15,16-Diepoxy-13(16),14-clerodadiene (1) and a new clerodane diterpenoid designated thysaspathone (2) were isolated from the liverwort *Thysananthus spathulistipus*, while *Radula appressa* produced radulannin A (3), radulannin L (4), 2-geranyl-3,5-dihydroxybibenzyl (5), 2(*S*)-2-methyl-2-(4-methyl-3-pentenyl)-7-hydroxy-5-(2-phenylethyl) chromene (*o*-cannabichromene) (6), 6-hydroxy-4-(2-phenylethyl) benzofuran (7), and *o*-cannabicyclol (8). All of the isolated compounds inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and the greatest inhibition was attributed to compound 5, with an IC_{50} value of 4.5 μM.

Key words liverwort; *Radula appressa*; *Thysananthus spathulistipus*; clerodane; prenylated bibenzyl; nitric oxide

Liverworts contain structurally interesting and biologically active terpenoids and aromatic compounds.[1)] The influence of geographic location as well as the environment on the chemical constituents of liverworts has been reported.[1,2)] However, chemical markers, which are important for the characterization of liverworts, are not affected although some derivatives could be produced due to the stress induced by the environment (climate, plants, bacteria, fungi, and animals). As part of our ongoing systematic investigation of the chemical constituents of Malagasy liverworts,[2—5)] *Thysananthus spathulistipus* (Lejeuneaceae) and *Radula appressa* (Radulaceae) have been studied. *Radula* species have been extensively investigated[6,7)] while only a few publications are available on species in the genus *Thysananthus*.[1)] The former are well known for their high prenylated bibenzyls contents and rearranged cannabinoids, while sterols and terpenoids have been reported in the latter species.[1,6—8)] The present paper deals with the isolation and structural determination of two clerodane diterpenes (**1**, **2**), prenylated bibenzyls, and their rearranged products (**3**—**8**) from *T. spathulistipus* and *R. appressa*, respectively, as well as their nitric oxide (NO)-inhibitory properties in lipopolysaccharide (LPS)-induced RAW 264.7 cells. To the best of our knowledge, this is the first report on the phytochemical investigation of the two liverwort species.

Results and Discussion

Size exclusion column chromatography on Sephadex LH-20 of the ether extract of *T. spathulistipus*, following by purification on silica gel and preparative HPLC afforded two clerodane diterpenes (**1**, **2**), while the *R. appressa* ether extract yielded radulannin A (**3**), radulannin L (**4**), 2-geranyl-3,5-dihydroxybibenzyl (**5**), 2(*S*)-2-methyl-2-(4-methyl-3-pentenyl)-7-hydroxy-5-(2-phenylethyl) chromene (*o*-cannabichromene, **6**), 6-hydroxy-4-(2-phenylethyl) benzofuran (**7**),[6,7)] and *o*-cannabicyclol (**8**).[9,10)] The structures of the new compounds were determined by careful interpretation of two-dimensional NMR spectroscopic data and CD spectral analysis, and those of the known compounds by comparison of their spectral data with those reported in the literature.

The molecular formula of compound **1** was determined to be $C_{20}H_{30}O_2$ in positive HR-EI-MS (a molecular ion peak at *m/z* 302.2245 $[M]^+$). The ^{1}H-NMR spectral data (Table 1) showed the presence of one secondary methyl and three quaternary methyl proton signals (δ 0.80, d, *J*=6.3 Hz; 0.66, 1.06, and 1.18, each a singlet), three proton signals of a β-substituted furan ring (δ 6.25, br m, 7.19, br m, and 7.34, t, *J*=1.6 Hz), together with a signal of H-3 of an α-oriented C-3—C-4 epoxide (δ 2.93, br s) of a *trans*-clerodane skeleton. The ^{13}C-NMR data (Table 2) coupled with the interpretation of the two-dimensional NMR data (including COSY, HMQC,

Table 1. ^{1}H-NMR Spectral Data for **1** and **2** (600 MHz, $CDCl_3$)

	1	2
1	1.27 (m)	1.39 (m)
2a	1.71 (m)	1.82 (m)
2b	2.14 (m)	2.24 (ddt, 14.3, 3.8, 1.9)
3	2.93 (br s)	3.04 (br s)
4		
5		
6a	1.40 (m)	2.46 (d, 14.0)
6b	1.65 (m)	2.46 (d, 14.0)
7a,b	1.48 (m)	
8	1.53 (m)	2.56 (q, 6.5)
9		
10	1.04 (dd, 10.7, 2.2)	1.68 (dd, 10.7, 2.2)
11a	1.49 (m)	1.54 (ddd, 14.3, 12.0, 4.9)
11b	1.60 (ddd, 14.3, 12.0, 5.4)	1.77 (m)
12a	1.48 (m)	2.35 (m)
12b	1.65 (br t, 13.4, 3.8)	2.35 (m)
13		
14	6.25 (br m)	6.26 (br m)
15	7.34 (t, 1.6)	7.37 (t, 1.6)
16	7.19 (br m)	7.23 (br m)
17	0.80 (d, 6.3)	0.96 (d, 6.5)
18	1.18 (s)	1.19 (s)
19	1.06 (s)	0.68 (s)
20	0.66 (s)	1.01 (s)

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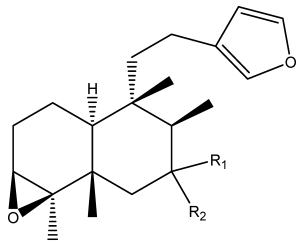
Table 2. ^{13}C-NMR Spectral Data for **1** and **2** (150 MHz, $CDCl_3$)

	1	**2**
1	15.3	15.7
2	28.1	27.8
3	62.1	62.0
4	66.4	65.3
5	37.2	44.2
6	37.1	53.1
7	28.2	212.1
8	36.0	50.0
9	39.1	41.4
10	47.9	47.7
11	38.6	39.1
12	18.3	18.8
13	135.5	124.5
14	110.9	110.7
15	142.7	143.0
16	138.3	138.5
17	16.0	8.1
18	19.7	19.5
19	16.8	19.6
20	18.5	17.8



1: $R_1 = R_2 = H$
2: $R_1 = R_2 = O$

Fig. 1. Structures of Compounds **1** and **2**

HMBC and NOESY) allowed us to determine the relative configuration of **1** to be 3α,4α:15,16-diepoxy-13(16),14-clerodadiene, previously isolated from *Solidago serotina*.[11] However, in the literature, the ^{1}H- and ^{13}C-NMR data of **1** have not been fully reported and the absolute configuration was not determined. The ^{1}H- and ^{13}C-NMR data of **1** (Tables 1, 2, respectively) are reported in the present communication. The absolute configuration (Fig. 1) is enantiomeric to that of the previously isolated compound, as corroborated by the positive value of its optical rotation ($[\alpha]_D^{20}$ +32°).

Positive-ion HR-EI-MS of compound **2** exhibited a quasi-molecular ion peak at *m/z* 316.2039 $[M]^+$, corresponding to the molecular formula $C_{20}H_{28}O_3$. The absorption bands at 2970, 2902, 1500, and 883 cm^{-1} and that at 1707 cm^{-1} in the IR spectrum suggest the presence of a furyl and a ketone in the molecule. Moreover, the ^{1}H-NMR spectrum which is very similar to that of **1**, showed signals due to C-17, C-18, C-19, and C-20 methyl protons [δ 0.96 (d, J=6.5 Hz, 1.19 (s), 0.68 (s), and 1.01 (s), respectively], resonance of an H-3 proton (δ 3.04, br s) of the α-oriented C-3—C-4-epoxide, and signals of the β-substituted furan protons [δ 6.26 (br m, H-14), 7.23 (br m, H-16), 7.37 (t, 1.6, H-15)]. The ^{13}C-NMR spectrum exhibited 20 signals attributable to a 3α,4α:15,16-diepoxy-ketoclerodane. On comparison of the ^{13}C-NMR spectral data of **2** with that of **1** (Table 2), differences due to the presence of a ketone (δ 212.1) at C-7 could be

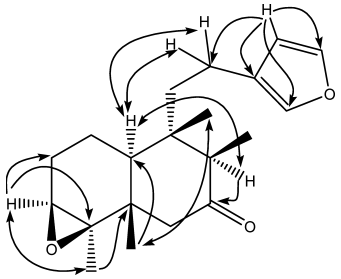


Fig. 2. Important NOE (Single Arrow) and HMBC (Double Arrow) Correlations Observed in **2**

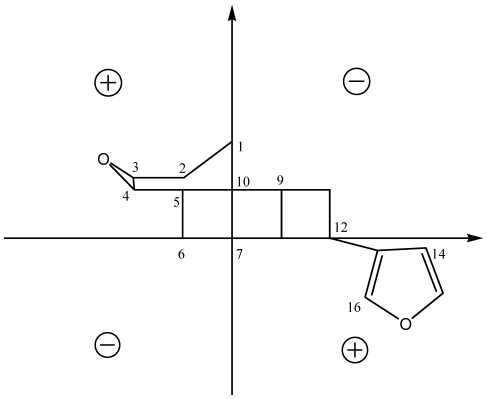


Fig. 3. Octant Projection Diagram for Compound **2**

observed in the C-6, C-8, and C-17 carbon signals. To establish the exact structure of **2**, two-dimensional NMR experiments including COSY, HSQC, and HMQC were carried out. In the COSY spectrum, the partial structures –CH_2–CH_2–CHO–CHO–, –CH_2–CH_2–, and a furanyl ring were seen. The location of the ketone group at C-7, the epoxide at C-3 and C-4, and the β-furanyl at C-12 were determined based on the HMBC correlations (Fig. 2) between H-3 (δ 3.04, br s) and C-2 and C-4; H-8 (δ 2.56, q) and C-7; and H-16 (δ 7.23, br m) and C-13, C-14, C-15, and C-12. Furthermore, the relative configurations of the methyl groups were corroborated by the observation of the NOE cross-peaks between CH_3-19 and CH_3-20 and between H-10 and H-8. The positive sign of the Cotton effect at 290 nm ($\Delta\varepsilon_{290}$=+1.8) observed in the CD spectrum could be predicted by the back octant rule to determine the absolute configuration of compound **2**. Due to the *trans*-fused decalin, the C-3—C-4 epoxide lies in the higher left (+) octant, while the furane ring was located in the lower right (+) octant, and the remaining substituents surrounding the carbonyl chromophore have symmetric partners, which therefore do not give weight to the Cotton effect (Fig. 3). The absolute configuration of thysaspathone (**2**) is thus concluded to be 3β,4β:15,16-diepoxy-13(16),14-clerodadiene-7-one, as shown in Fig. 1.

Clelodane-type diterpenoids have mainly been found in the Jungermanniaceae, particularly in the subfamilies Lophozioideae and Jungermannioideae. The distribution of clerodane-type diterpenoids among Lejeuneaceae is very rare. As far as we are aware, only one example of the isolation of *ent*-cleroda-13,14-dien-13ξ-ol [=(−)-kolavelool] has been reported from *Macrolejeunea pallescens*.[12] The presence of compounds enantiomeric to those found in higher

Table 3. Effect of Compounds **1**—**8** on NO Production by LPS-Induced RAW 264.7 Macrophages

Compounds	IC_{50} (μM)
1	20.1
2	11.6
3	20
4	15.3
5	4.5
6	16.1
7	12.7
8	13.2
(L-N^6-1-Iminoethyl)lysine	18.6

plants in the liverworts has been reported.[1)] The present findings also indicate that both clerodane (**1**, **2**) and *ent*-clerodane [(−)-kolavelool] could be found in the Lejeuneaceae family. Their roles in the producing organism are not yet known, although it is obvious that they act as chemical defense against predators. To evaluate the activity of the isolated compounds and determine whether the enantiomeric compounds have biological activity, their inhibition of NO production in LPS-stimulated RAW 264.7 cells was evaluated.

Finding new agents that inhibit NO production from natural sources is important in drug discovery, since overproduction of NO by inducible nitric oxide synthase (iNOS) is involved in inflammatory disease.[13,14)] Compounds **1**—**8** were examined for inhibition of NO production in culture media of RAW 264.7 cells in response to LPS. All of the compounds showed inhibition similar to or stronger than that of (L-N^6-1-iminoethyl)lysine, used as a control[15)] (Table 3). The greatest inhibition was attributed to 2-geranyl-3,5-dihydroxybibenzyl (**5**, IC_{50} 4.5 μM), which was isolated in high amounts from *R. appressa*. Thysaspathone (**2**, IC_{50} 11.6 μM) exhibited moderate inhibition, while its C-7 deoxo derivative (**1**, IC_{50} 20.1 μM) showed weak activity. The photochemical reaction product, compound **8**, displayed stronger activity than its precursor (**6**).[9,10)] The strong inhibition of the prenylated bisbibenzyls and bibenzyl-cannabinoids (**3**—**8**, Fig. 4) was suggested to be due to their antioxidant properties. Further investigation is needed to determine the mechanism of inhibition of NO production by compounds **1**—**8**.

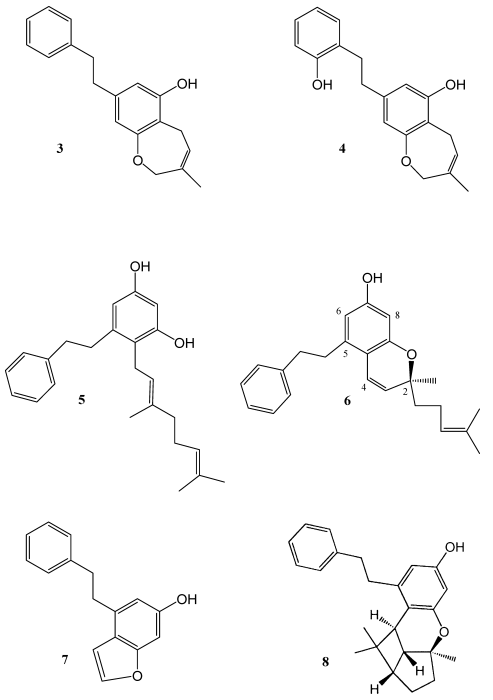


Fig. 4. Structures of Compounds **3**—**8**

Experimental

General Procedures Optical rotations were measured on a Jasco DIP-1000 polarimeter with MeOH as a solvent. UV spectra were obtained on a Shimadzu UV-1650PC instrument in MeOH. IR spectra were measured on Perkin Elmer Spectrum One FT-IR Spectrometer. 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 $CDCl_3$ as a solvent. Chemical shifts are given relative to TMS (δ 0.00) as an internal standard (^{1}H) and δ 77.0 (ppm) from $CDCl_3$ as a standard (^{13}C). Mass spectra were recorded on a JEOL JMS AX-500 spectrometer. Column chromatography was carried out on a Sephadex LH-20 column (Amersham Pharmacia Biotech, CH_2Cl_2–MeOH 1 : 1 as a solvent system) and silica gel (Kieselgel 60: 0.040—0.063 mm, Merck). The preparative HPLC experiment was performed using a Cosmosil reverse-phase column, Jasco 880-PU pump, Jasco 875-UV UV detector, and ERC-7512 (Erma CR, Inc), RI detector.

Plant Materials *R. appressa* and *T. spathulistipus* were collected in Moramanga, Madagascar, in March 2004 and identified by Mr. M. Wigginton of Tropical Bryology Research (London and Peterborough, U.K.). Voucher specimens were deposited in the Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

Extraction and Isolation Powdered *T. spathulistipus* (5.2 g) and *R. appressa* (21.0 g) were extracted with ether at room temperature for 1 month. Each extract was filtered and concentrated *in vacuo* to yield 400.0 mg of green oils from *T. spathulistipus* and 867.0 mg from *R. appressa*. The former extract was divided into five fractions using size-exclusion chromatography (Sephadex LH-20). Fraction 3 was chromatographed on a silica gel column to afford 11 subfractions. Fraction 3-2 afforded compound **1** (3.67 mg), while compound **2** (7.8 mg) was obtained from fraction 3-8.

The greenish oil extract of *R. appressa* was fractionated on Sephadex LH-20 column chromatography to afford four fractions. Fraction 3 was further divided into 10 subfractions on silica gel column chromatography (solvent system: hexane : ethyl acetate; 10% to 100% EtOAc). HPLC (80% MeOH) of fraction 3-4 yielded compound **6** (3.2 mg), while compounds **3** (4.3 mg) and **8** (6.2 mg) were obtained from fraction 3-5. Compound **7** (1.6 mg) was isolated from fraction 3-6 by ODS HPLC (80% MeOH). Fraction 3-8 was subjected to ODS column chromatography (aqueous MeOH 80% to 100% MeOH) to give compounds **4** (8.2 mg) and **5** (40.3 mg).

3β,4β:15,16-Diepoxy-13(16),14-clerodadiene (**1**): Amorphous powder, $[\alpha]_D^{20}$ +32° (c=0.7, MeOH). Positive HR-EI-MS: m/z 302.2245 $[M]^+$, $C_{20}H_{30}O_2$, requires 302.2244. IR (KBr) cm^{-1}: 2990, 2970, 1489, and 880. ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

Thysaspathone (**2**): Amorphous powder, $[\alpha]_D^{20}$ −36.3° (c=2.3, MeOH). Positive HR-EI-MS: m/z 316.2039 $[M]^+$, $C_{20}H_{28}O_3$, requires 316.2039. UV λ_{max} (MeOH) nm (log ε): 209 (4.2), 290 (1.7). IR (KBr) cm^{-1}: 2970, 2902, 1707, 1500, 883. ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

Cell Culture RAW 264.7 cells, a mouse macrophage cell line, were grown in RPMI 1640 supplementented with 10% fetal bovine serum, kanamycin (50 μg/ml), and ampicillin (60 μg/ml) at 37 °C under an atmosphere of 5% CO_2 and 95% air.

Inhibition of NO Production by 1—8 RAW-264.7 cells 100 μl (8×10^5 cells/ml) were pipetted into each well and cultured in 96-well culture plates. After 24 h incubation, 50 μl of medium containing each compound previously diluted in DMSO at different concentrations (40, 30, 20, 10, 5, 2.5 μM; the final DMSO concentration was less than 0.1%, and at this concentration DMSO did not show any NO induction without LPS stimulation) was added to each well. Then, 50 μl of vehicle or LPS purified from *Pantoea agglomerans* (4 μg/ml) was added. The cells were further incubated at 37 °C for 24 h. The supernatant (35 μl) of each well was collected, mixed with Griess reagents[16)] (35 μl), and their absorbance at 550 nm was measured using a Bio-Rad model 550 microplate reader. The NO concentration was determined by measuring the amount of nitrite in the cell culture supernatant using Griess reagents, and IC_{50} values were calculated as described in Table 3.

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