Chemical Constituents of Malagasy Liverworts. 6. A Myltaylane Caffeate with Nitric Oxide Inhibitory Activity from *Bazzania nitida*

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The phytochemical investigation of the Malagasy liverwort, *Bazzania nitida*, led to the isolation of (+)-(l*S*,4*R*)-7-hydroxycalamenene (**1**), together with a new myltayl-4(12)-ene-2-caffeate (**2**). Although cyclomyltaylane sesquiterpenoids have been known to be present in *Bazzania* species, this is the second isolation of myltaylane-type sesuiterpenoids from the genus. Biosynthetically, myltaylane sesquiterpenoids are proposed to be derived from chamigrene with a route different from those of cyclomyltaylanoids. The chemosystematics of *Bazzania* species is also discussed. Compound **2** showed potent inhibition of nitric oxide in LPS-induced RAW 264.7 cells (IC_{50} = 6.3 μM).

Malagasy liverworts produce novel and known secondary metabolites with chemotaxonomical value and pharmacological interest.[1−5] Among liverworts, *Bazzania* species are unique since they contain sesquiterpene caffeates, which have a wide range of biological activities such as cytotoxic, antimicrobial, antifungal, and superoxide anion release inhibitory activity.[6−8] In our previous reports on chemical studies of Malagasy *Bazzania* species, we reported the isolation of isobicyclogermacrenal, drimenol, *cis*- and *trans*-drimenyl caffeates, 2-methoxy-5-hydroxycuparene (HM-1), 2-methoxy-4-methoxycuparene, and friedelin from *B. decrescens* and (+)-globulol, *ent*-4β,10α-dihydroxyaromadendrane, 1*R*,5*R*-diacetoxycyclomyltaylan-10-one, 5*R*-acetoxy-1*R*-hydroxycyclomyltaylan-10-one, 1*R*,5*R*-dihydroxycyclomyltaylan-10-one, 5*R*,10β-diacetoxycyclomyltaylan-9β-ol, 5*R*,10β,13-triacetoxycyclomyltaylan-9β-ol, 5*R*,9β,13-triacetoxycyclomyltaylan-10β-ol, and 1*S**,4*S**,5*S**-acora-8(15),9-dien-7*R**-ol from *B. madagassa*.[3,5] The inhibition of iNOS mRNA expression in LPS-induced RAW 264.7 cells of 2-hydroxy-4-methoxycuparene isolated from *Bazzania* has been carried out and was shown to be dose dependent.[9] In continuation of our phytochemical investigation of the chemical constituents of Malagasy liverworts as well as the biological activities of their metabolites, we have isolated a known calamename-type sesquiterpenoid and myltayl-4(12)-ene-2-caffeate from *B. nitida*. The present paper discusses the isolation and the structure elucidation of the new and known compounds, the NO production inhibition properties of the new compound in LPS-induced RAW 264.7 cells, and the chemosystematics of *Bazzania* species.

A combination of size exclusion (Sephadex LH-20) and silica gel column chromatography, and ODS-HPLC of the ether extract of *B. nitida*, afforded (+)-(l*S*,4*R*)-7-hydroxycalamenene (**1**) and myltayl-4(12)-ene-2-caffeate (**2**). Compound **1** was identified by comparison of its NMR data and specific rotation ($[\alpha]_D^{20}$ +55.4, *c* 0.6, $CHCl_3$) with those of (+)-(l*S*,4*R*)-7-hydroxycalamenene, previously isolated from the cultured cells of *Heteroscyphus planus*.[10]

Compound **2** exhibited a molecular formula of $C_{24}H_{30}O_4$, as determined by HREIMS (*m*/*z* 382.2152 $[M]^+$, requires 382.2144). Its IR spectrum showed absorption bands characteristic for hydroxy groups (3318 cm^{-1}), aromatic methine strechings (3060 and 1028 cm^{-1}), and a conjugated carbonyl at 1681 cm^{-1}. The ^1H NMR data (Table 1) showed the presence of *trans*-olefin protons (δ 6.25, d, J = 15.9 Hz and δ 7.55, d, J = 15.9 Hz) and an ABX system (δ 6.80, d, J = 8.2 Hz; 7.01, dd, J = 8.2, 1.9 Hz and δ 7.10, d, J = 1.9 Hz) corresponding to a caffeoyl moiety, together with three quaternary carbon attached methyl proton resonances (δ 0.81, 1.02, 1.22, each singlet), an exomethylene (δ 4.75 and 4.98, each doublet J = 1.3 Hz), an oxygen-bearing methine proton (δ 4.89, dd, J = 7.6, 3.8 Hz), and resonances for methine and methylene protons. The ^{13}C NMR data of **2** displayed 24 carbon signals, nine of which were ascribable to the caffeoyl moiety and the remaining 15 to a tricyclic sesquiterpene aglycone with an exomethylene as determined by DEPT data (Table 1). The three partial structures, $-CH_2-CH_2-CH_2-$ (1), $-CH_2-CH(O)-CH-$ (2), and $CH_2=C-CH-$ (3), observed in COSY and the three quaternary methyl and four quaternary carbon resonances suggested the presence of a myltaylane sesquiterpene alcohol. HMBC and NOESY experiments were performed to establish the location of the functional groups and the full structure of **2** (Figure 1). The exomethylene was located at C-4 due to the long-range correlation observed between H-12ab and C-5 and C-3. The cross-peaks between the oxymethine proton (δ 4.89) and C-1, C-3, C-6, and the carbonyl carbon (δ 167.0) substantiated the location of the caffeoyl group at C-2. The pseudoaxial orientation of H-2 was corroborated by the coupling pattern of H-3 (δ 2.42, s), which did not show any coupling with

Table 1. ^1H and ^{13}C NMR Data for **2** (600 and 150 MHz, respectively)

position	H	C	(DEPT)
1a, b	1.96 m	38.1	(t)
2	4.89 dd (7.6, 3.8)	77.8	(d)
3	2.42 s	62.7	(d)
4		158.7	(s)
5a	11.80 dt (17.0, 1.3)	39.1	(t)
5b	2.52 brd (17.0)		
6		53.9	(s)
7		47.0	(s)
8a	1.22 m	30.1	(t)
8b	1.38 td (13.6, 3.8)		
9a	1.48 m	18.5	(t)
9b	1.73 dt (13.6, 4.1)		
10a	1.21 m	35.7	(t)
10b	1.54 dd (13.6, 4.4)		
11		33.3	(s)
12a	4.75 d (1.3)	105.6	(t)
12b	4.98 d (1.3)		
13	1.22 s	20.1	(q)
14	1.02 s	23.2	(q)
15	0.81	28.6	(q)
caffeoyl-C=O		167.0	(s)
α	6.25 d (15.9)	116.2	(d)
β	7.55 d (15.9)	144.4	(d)
1′		127.6	(s)
2′	7.10 d (1.9)	114.3	(d)
3′		146.1	(s)
4′		148.2	(s)
5′	6.80 d (8.2)	115.5	(d)
6′	7.01 dd (8.2, 1.9)	122.3	(d)

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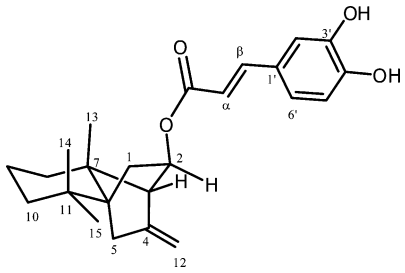

Figure 1. Structure of compound **2**.

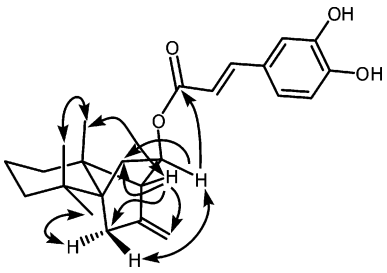

Figure 2. Important HMBC (arrow) and NOE (double arrows) correlations observed for **2**.

H-2, and the NOESY correlation (Figure 2) observed between H-2 and H-5a (δ 1.80). The absolute configuration could not be determined, but it could be assumed to be the same as those of the previously isolated myltaylane sesquiterpenes from *Bazzania* and *Mylia* species.[6] From the above data the structure of **2** was determined to be myltayl-4(12)-ene-2-caffeate.

Caffeates have been shown to have anti-inflammatory effects by inhibition of prostaglandin E_2, nitric oxide, and TNF-α production. The mechanisms were suggested to be from the inhibition of iNOS, COX-2, and TNF-α expression through the down regulation of NF-κB binding activity.[11] Compound **2** was tested for its nitric oxide inhibition activity in LPS-induced RAW 264.7 cells. Strong inhibition of NO production ($IC_{50} = 6.3\ \mu M$) was observed. This result supported the speculation that esterification of caffeic acid with lipophilic sesquiterpene alcohols facilitated its transport into cells, and once penetrated, compounds react to display inhibition effects.[12]

The results of the examination of the chemical constituents of three Malagasy *Bazzania* species, *B. decrescens*, *B. madagassa*, and *B. nitida*, showed that *B. decrescens* is very different from the two species since it contains drimenol and drimenyl caffeate, together with cuparene-type sesquiterpenoids. *B. madagassa* is characterized by its high content of cyclomyltaylane-type sesquiterpenoids, which are reported to be biosynthesized via chamigrene.[3] Interestingly, a GC-MS chromatogram of *B. decrescens* did not show the peak of chamigrene, which was observed in *B. madagassa* and *B. nitida*. Although only two compounds (**1**, **2**) could be isolated from *B. nitida* in the present study, GC-MS analysis of the ether extract revealed the presence of β-barbatene (3.6%), isobazzanene (10.3%), gimnomitrol (1.2%), chamigrene (4.8%), and acora-3,5 diene (1.7%). Investigation of the French *B. trilobata* revealed the presence of a myltaylene alcohol (**3**), which was the first myltaylane-type sesquiterpene isolated from *Bazzania* species.[13] The phytochemical exploration of *B. madagassa*, *B. japonica*, and *B. tridens* led to the isolation of cyclomyltaylane sesquiterpenes, while the French species did not contain a trace.[3,5,13−15] Known myltaylane

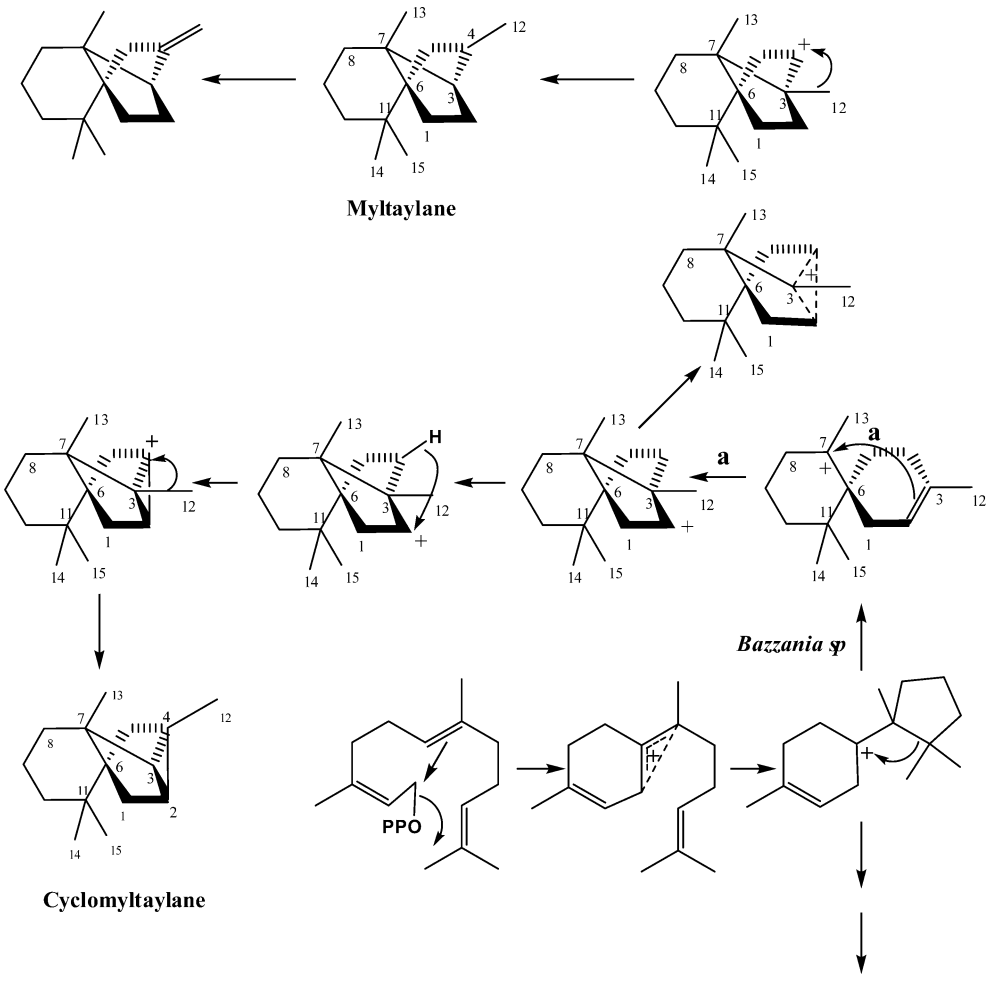

Figure 3. Plausible biosynthetic pathway of myltaylane and cyclomyltaylane in *Bazzania species*.

and cyclomyltaylane sesquiterpenes have the same configuration at C-3, C-6, and C-7. Therefore, the biosynthetic pathway of myltaylane and cyclomyltaylane sesquiterpenes in *Bazzania* species from two different routes is proposed in Figure 3. Noteworthy, cyclomyltaylanes and myltaylanes do not occur at the same time in one plant. This fact combined with the previous results[6] suggested that the two enzymes responsible for their bisosynthesis are not present at the same time in one *Bazzania* species. Further investigation of biosynthesis with ^{13}C-labeled substances is needed to confirm the biosynthetic routes.

Bazzania species have been classified in two chemotypes: albicanyl (drimenyl) caffeate-couparane-type (type I) and a calamenane-type (type II).[6] The latter could be found in *B. nitida* and *B. trilobata*, which contain myltaylanes. It is interesting to note that cyclomyltaylane-containing *Bazzania* species do not show the presence of calamenane. *B. japonica* contains cyclomyltaylanes together with albicanyl caffeate, allowing the classification of the plant as type I.[14] *B. madagassa*, however, is rich in cyclomyltaylane-type sesquiterpenes but does not contain albicanyl (drimenyl) caffeate. Therefore, *Bazzania* species containing cyclomyltaylane or myltaylane sesquiterpenes can be classified as types I and II.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 polarimeter with $CHCl_3$ as solvent. IR spectra were measured on a Perkin-Elmer Spectrum One FT-IR spectrometer. The ^1H and ^{13}C NMR spectra were recorded on a Varian Unity 600 NMR spectrometer (600 MHz for ^1H 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 (^1H) 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 Sephadex LH-20 (Amersham Pharmacia Biotech, CH_2Cl_2−MeOH, 1:1, as the solvent system) and silica gel (Kieselgel 60: 0.040−0.063, Merck). The preparative HPLC experiment was performed using a Cosmosil reversed-phase column, JASCO 880-PU pump, JASCO 875-UV UV detector, and ERC-7512 Erma CR Inc. RI detector. The gas chromatography−mass spectroscopy (GC-MS) analysis was performed on a Hewlett-Packard HP5890 series GC system, equipped with 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}). The temperature programming of the GC-MS analysis was performed from 50 °C, then 50−250 °C at 15 °C min^{-1}, and finally isothermal at 250 °C. Mass spectra were measured at 70 eV. HRFABMS were recorded on a JEOL JMS AX-500.

Plant Material. *B. nitida* (Lepidoziaceae subf. Lepidoziodeae) was collected in Moramanga, Madagascar, in June 2005 by L.H. and identified by Mr. M. Wigginton of Tropical Bryology Research (London and Peterborough, UK). Voucher specimens (MAD20054) were deposited in the Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

Extraction and Isolation. The powdered *B. nitida* (9 g) was extracted with ether at room temperature for 1 month. The extract was filtered and concentrated *in vacuo* to yield 800 mg of green oil, which was divided into four fractions based on size-exclusion chromatography (Sephadex LH-20). Fraction 2 was subjected to silica gel column chromatography (solvent systems: hexane−EtOAc, 9:1; 1:1; and 100% EtOAc) to give compound **1** (3.8 mg). Fraction 3 was applied to silica gel column chromatography using hexane and EtOAc (4:1 to 100% EtOAc) as solvent to afford four subfractions. Preparative ODS-HPLC (90% MeOH) of fraction 3-3 afforded compound **2** (2.2 mg).

Myltayl-4(12)-ene-2-caffeate (2): oil, $[\alpha]_D^{20}$ +2.4 (*c* 0.9, $CHCl_3$); positive HREIMS *m*/*z* 382.2152 $[M]^+$, $C_{24}H_{30}O_4$, requires 382.2144; IR ν_{max} 3318, 3060, 1681, 1028 cm^{-1}; ^1H and ^{13}C NMR, see Table 1.

Bioassays. Cell Culture and Sample Treatment. RAW 264.7 cells were cultured in RPMI medium containing 10% FBS, kanamycin (50 μg/mL), and ampicillin (60 μg/mL). Cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air. For the screening, 20 μM compound **2** was solubilized in EtOH. The final EtOH concentration was below 0.1% in the culture plate. At this concentration, EtOH did not show NO induction without stimulation with LPS. Cells were incubated with compound **2** at 20 μM and stimulated with LPS at 4 μg/mL for 24 h.

Nitrite Assays. Nitrite, accumulated in the culture medium, was measured as an indicator of NO production by the Griess reaction [cell culture medium (35 μL) was mixed with Griess reagent (35 μL), prepared by addition of equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) H_3PO_4 and 0.1% (w/v) naphthylethylenediamine HCL] and incubated at room temperature for 10 min. The absorbance was then measured at 550 nm using a microplate reader. In all experiments, fresh culture medium was used as control. The amount of nitrite in **2** was calculated by means of the $NaNO_2$ serial dilution standard curve freshly prepared. The nitrite concentration produced by the LPS without sample treatment was used as positive control, while the negative control was that of sample without LPS stimulation. The IC_{50} values were calculated from the results of the NO inhibition and were 20.0, 10, 5, 2.25, 1.12, and 0.5 μM for compound **2**.

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Supporting Information Available: The structures of compounds **1** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Harinantenaina, L.; Asakawa, Y. *Biochem. Syst. Ecol.* **2004**, *32*, 1073−1078.
(2) Harinantenaina, L.; Asakawa, Y. *Chem. Pharm. Bull.* **2004**, *52*, 1382−1384.
(3) Harinantenaina, L.; Kurata, R.; Asakawa, Y. *Chem. Pharm. Bull.* **2005**, *53*, 515−518.
(4) Harinantenaina, L.; Takahara, Y.; Nishizawa, T.; Kohchi, C.; Soma, G.-I.; Asakawa, Y. *Chem. Pharm. Bull.* **2006**, *54*, 1046−1049.
(5) Harinantenaina, L.; Kurata, R.; Takaoka, S.; Asakawa, Y. *Phytochemistry* **2006**, *67*, 2616−2622.
(6) Asakawa, Y. In *Progress in the Chemistry of Organic Natural Products*; Herz W., Kirby G. B., Moore R. E., Steglich W., Tamm, Ch., Eds.; Springer: Vienna, 1995; Vol. 65, pp 1−618.
(7) Asakawa, Y. *Phytochemistry* **2004**, *65*, 623−669.
(8) Burgess, E. J.; Larsen, L.; Perry, N. B. *J. Nat. Prod.* **2000**, *63*, 537−539.
(9) Harinantenaina, L.; Quang, D. N.; Nishizawa, T.; Hashimoto, T.; Kohchi, C.; Soma, G.-I.; Asakawa, Y. *Phytomedicine*, in press.
(10) Nabeta, K.; Katayama, K.; Nakagawara, S.; Katoh, K. *Phytochemistry* **1993**, *32*, 117−122.
(11) Shin, K.-M.; Kim, I. T.; Park, Y.-M.; Ha, J.; Choi, J.-W.; Park, H.-J.; Lee, Y. S.; Lee, K.-T. *Biochem. Pharm.* **2004**, *68*, 2327−2336.
(12) Grunberger, D.; Banerjee, R.; Eisinger, K.; Oltz, E. M.; Efros, L.; Caldwell, M.; Estevez, V.; Nakanishi, K. *Experientia* **1988**, *44*, 230−232.
(13) Nagashima, F.; Momosaki, S.; Watanabe, Y.; Takaoka, S.; Huneck, S.; Asakawa, Y. *Phytochemistry* **1996**, *42*, 1361−1366.
(14) Asakawa, Y.; Toyota, M.; Ueda, A.; Tori, M.; Fukazawa, Y. *Phytochemistry* **1991**, *30*, 3037−3040.
(15) Wu, C. L.; Chang, S.-J. *Phytochemistry* **1992**, *31*, 2153−2154.

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