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Chemical constituents of Malagasy liverworts: Cyclomyltaylanoids from *Bazzania madagassa* ☆

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Abstract

Five cyclomyltaylanoids (**2**–**6**), together with 1*R*,5*R*-diacetoxycyclomyltaylan-10-one (**1**), (+)-globulol, and *ent*-4β,10α-dihydroxyaromadendrane have been isolated from the diethyl ether fraction of the Malagasy liverwort, *Bazzania madagassa*. The structure of **1** was confirmed by X-ray analysis, while those of the compounds were established on the basis of one- and two-dimensional NMR spectroscopic evidence, and comparison with data reported in the literature. The chemosystematics of *B. madagassa* are discussed.

Keywords: Liverwort; Lepidoziaceae subf. Lepidoziodeae; *Bazzania madagassa*; Cyclomyltaylane; Chemosystematics

1. Introduction

The isolation of structurally and biologically interesting terpenoids and aromatic compounds from liverworts incited us to investigate the chemical constituents of hepatics growing widely in Madagascar, which has very rich flora (Asakawa, 1995, 2004; Harinantenaina and Asakawa, 2004a,b; Harinantenaina et al., 2005). *Bazzania madagassa* is a leafy liverwort widely growing in the Eastern part of Madagascar and is often found at the base of trees or on rotten logs. The plants from this genus are very rich in sesquiterpenoids and aromatic compounds, such as lignans and bisbibenzyls (Asakawa et al., 1991; Asakawa, 1995, 2004; Martini et al., 1998a,b; Scher et al., 2003). Furthermore, among the liverworts, *Bazzania* is the only genus containing sesquiterpene caffeates, which have been shown to have useful activities such as cytotoxic, antimicrobial, antifungal, and superoxide anion release inhibitory activity (Asakawa, 1995, 2004; Burgess et al., 2000). Cyclomyltaylanoids are tetracyclic sesquiterpenoids first isolated from the liverwort *Mylia taylorii* (Takaoka et al., 1985, 1988). This group of compounds has been detected in the genus *Bazzania* although the latter do not have any morphological affinity with *Mylia* (Asakawa et al., 1991; Wu and Chang, 1992). The isolation of a new cyclomyltaylane sesquiterpenoid (**1**) and 1*S**,4*S**,5*S**, acora-8(15),9-dien-7*R**-ol from *B. madagassa* collected in Moramanga/Andasibe-Madagascar (voucher number: 2003LIV5) prompted us to investigate a larger amount of sample collected in Moramanga/Lakato (2003LIV3) (Harinantenaina et al., 2005). Five new cyclomyltaylanoids (**2**–**6**), together with 1*R*,5*R*-diacetoxycyclomyltaylan-10-one (**1**), (+)-globulol (Toyota et al., 1999), and *ent*-4β,10α-dihydroxyaromadendrane (Nagashima et al., 1994) were isolated. This paper deals with the isolation and structure elucidation of compounds **1**–**6** and comparison of the two sample collections.

2. Results and discussion

The total ion chromatogram obtained from GC/MS of the diethyl ether extract was very similar to that of the previous sample (2003LIV5), except for the presence of a few

☆ Part IV. For Part III, see: Harinantenaina et al. (2005).

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peaks which could not be identified by our GC/MS library. Only a small difference was observed in the amount of the identifiable constituents of the present and the former specimens: β-barbatene (0.6/5.8%), isobazzanene (1.7/9.2%), β-chamigrene (3.1/6.8%), δ-cuparene (7.5/16.2%), and acora-3,5 diene (4.4/1.2%). Moreover, in order to get more information about the cyclomyltaylanoid content and the other chemical constituents of *B. madagassa*, a combination of size exclusion and silica gel column chromatography of the remaining diethyl ether extract was performed to afford eight compounds, of which five were new (**2**–**6**, Fig. 1). The NMR spectra, CD spectral data, and the optical rotation of **1** were identical to the previously isolated compound from the liverwort sample 2003LIV5. The absolute configuration was determined by the observation of the negative Cotton effect ($\Delta\varepsilon_{298} - 12$) in the CD spectrum as determined previously (Asakawa et al., 1991) and the *R* configuration of C-1 and C-5 was substantiated by NOESY correlations between H-5 and H-8b and between C-1 acetyl methyl and C-14 and C-7 methyl groups (Harinantenaina et al., 2005). This time **1** was obtained in crystalline form (from 1:1 MeOH/Et_2O) and its structure was confirmed by X-ray analysis. The ORTEP drawing is shown in Fig. 2. The two other known compounds isolated were identified as (+)-globulol, and *ent*-4β,10α-dihydroxyaromadendrane, respectively, by comparison of their physical and spectroscopical data with those reported in the literature.

The molecular formula of compound **2** was determined as $C_{17}H_{24}O_4$ by HREIMS. The IR spectrum showed the presence of a ketone, an ester carbonyl, and a hydroxyl group at ν_{max} 1690, 1732, and 3507 cm^{-1}. The ^1H NMR spectrum exhibited signals of four quaternary methyl groups at δ: 1.01, 1.07, 1.40, and 1.69, an acetyl methyl at 2.03, together with signals of two oxygen-bearing methines (δ_H 4.76, *brs*, H-1, and δ_H 5.30, *s*, H-5). The ^{13}C NMR spectroscopic data displayed two signals for an acetate group (δ_C 170.6 and 21.6) and 15 carbon resonances for a sesquiterpene moiety. Inspection of its ^1H and ^{13}C

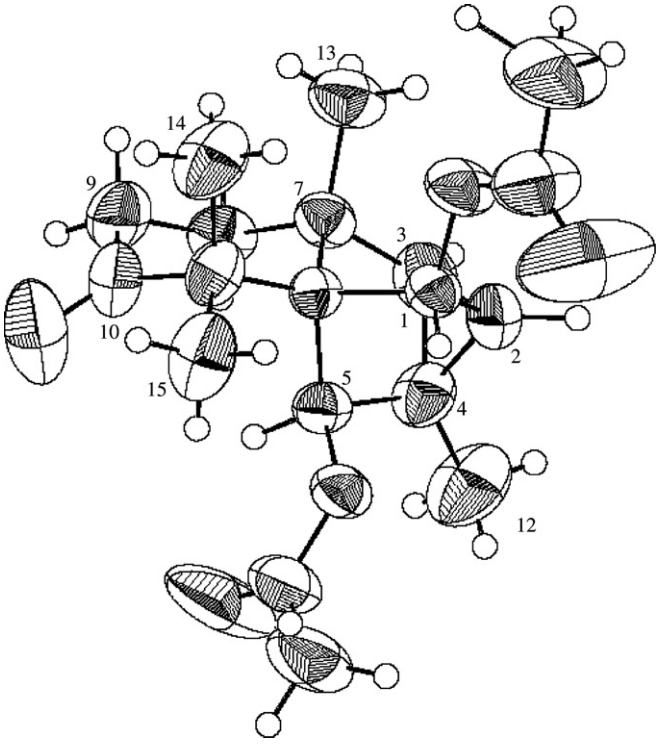
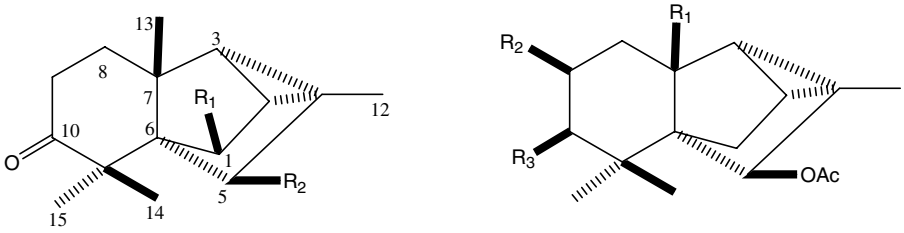
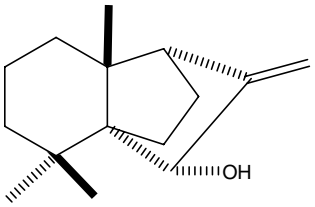


Fig. 2. ORTEP drawing of **1**.



1: R_1= $OCOCH_3$, R_2= $OCOCH_3$
2: R_1= OH, R_2= $OCOCH_3$
3: R_1= OH, R_2= OH

4: R_1= CH_3, R_2= OH, R_3= $OCOCH_3$
5: R_1= CH_2OCOCH_3, R_2= OH, R_3= $OCOCH_3$
6: R_1= CH_2OCOCH_3, R_2= $OCOCH_3$, R_3= OH



7

Fig. 1. Structures of cyclomyltaylanoids isolated from *Bazzania* species.

NMR spectroscopic data (Tables 1 and 2) revealed that compound **2** is very similar to **1**, except for the absence of the signal of an acetate group in **2**. Comparison of the ^{13}C NMR spectroscopic data of **1** and **2** suggested that deacetylation had occurred at C-1. The signal due to C-1 of **1** shifted upfield (−0.6 ppm), while downfieldshifts were observed in those due to C-2 and C-6 (+2.8 ppm and +1.0 ppm, respectively; Table 2). Moreover, a cross-peak observed between the methyl group at δ_H 5.30 and the acetyl carboxyl carbon (δ_C 170.6) in the HMBC spectrum confirmed the attachment of the acetate group to be at C-5 (Fig. 3). The location of the hydroxyl group at C-1 was

Table 1
^{1}H NMR spectroscopic data for **2**–**6** (600 MHz, in $CDCl_3$)

C	2	3	4	5	6
1a	4.76 *brs*	4.70 *d* (1.0)	1.66 *dd* (11.0, 1.3)	1.70 *dd* (11.0, 1.3)	1.65 *dd* (11.0, 1.3)
1b			1.46 *d* (11.0)	1.53 *d* (11.0)	1.57 *d* (11.0)
2	1.38 *brd* (*J* = 5.2)	1.36 *brd* (5.2)	1.06 *brd* (5.2)	1.15 *brd* (5.2)	1.15 *brd* (5.2)
3	1.39 *d* (*J* = 5.2)	1.33 *d* (5.2)	1.01 *d* (5.2)	1.42 *d* (5.2)	1.42 *d* (5.2)
4	–	–	–	–	–
5	5.30 *s*	3.87 *s*	5.00 *s*	5.00 *s*	5.01 *s*
6	–	–	–	–	–
7	–	–	–	–	–
8a	1.73 *ddd* (14.0, 7.4, 2.0)	1.67 *ddd* (14.0, 7.4, 2.0)	2.40 *dd* (14.5, 3.8)	2.26 *m*	2.26 *m*
8b	2.03 *ddd* (14.0, 13.1, 5.7)	1.88 *ddd* (14.0, 13.1, 5.7)	1.87 *dd* (14.5, 2.2)	2.26 *m*	2.26 *m*
9a	2.32 *ddd* (15.4, 13.1, 7.4)	2.81 *ddd* (15.4, 13.1, 7.4)	4.16 *td* (3.8, 2.2)	4.16 *td* (3.8, 3.8)	5.24 td (4.3, 3.2)
9b	2.75 *ddd* (15.4, 5.7, 2.0)	2.23 *ddd* (15.4, 5.7, 2.0)			
10	–	–	5.28 d (3.8)	5.29 d (3.8)	3.88 *d* (4.3)
11	–	–	–	–	–
12	1.01 *s*	1.12 *s*	1.03 *s*	1.06 *s*	1.05 *s*
13a	1.69 *s*	1.69 *s*	0.81 *s*	3.95 *d* (9.6)	3.74 *d* (9.8)
13b				5.05 *d* (9.6)	4.87 *d* (9.8)
14	1.07 *s*	1.28 *s*	1.14 *s*	1.01 *s*	1.00 *s*
15	1.40 *s*	1.42 *s*	1.34 *s*	0.83 *s*	1.01 *s*
5-CH_3C=O	2.03 *s*		2.16 *s*	2.18 *s*	2.07 *s*
9-CH_3C=O					2.08 *s*
10-CH_3C=O			2.13 *s*	2.13 *s*	
13-CH_3C=O				2.11 *s*	2.10 *s*

Table 2
^{13}C NMR spectroscopic data for **2**–**6** in $CDCl_3$

C	1	2	3	4	5	6
1	77.5	76.9 (−0.6)	76.5	28.1	27.9	28.2
2	26.0	28.8 (+2.8)	28.7	18.8	18.9	19.0
3	34.8	35.4	35.3	35.2	30.1 (−5.1)	30.4
4	23.8	23.5	24.4 (+0.9)	22.7	22.4	22.9
5	82.5	83.6	82.7 (−0.9)	85.6	85.4	85.5
6	56.4	57.4 (+1.0)	57.7 (+0.3)	54.6	55.6	55.0
7	46.2	45.9	45.2	44.9	48.5 (+3.6)	48.1
8	32.5	33.6	33.7	36.6	30.8 (−5.8)	28.8 (−2.0)
9	33.3	33.1	33.0	70.0	69.6	73.9 (+4.3)
10	214.7	215.8	217.0	77.5	76.7	73.8 (−2.9)
11	47.7	48.2	48.7	36.2	36.0	36.9
12	13.0	13.4	12.8	12.7	12.6	12.6
13	24.2	24.5	24.6	25.6	69.9	69.0
14	22.4	22.8	22.9	20.7	19.8	19.8
15	24.6	25.3	25.1	25.1	25.3	25.7
1-CH_3C=O	21.4					
1-CH_3C=O	170.5					
5-CH_3C=O	21.7	21.6		21.6	21.2	21.4
5-CH_3C=O	170.4	170.6		170.4	170.5	171.0
9-CH_3C=O						20.9
9-CH_3C=O						171.3
10-CH_3C=O				21.0	21.0	
10-CH_3C=O				170.5	171.6	
13-CH_3C=O					21.0	21.0
13-CH_3C=O					171.6	171.6

Values in parenthesis represented the upfield or downfield shifts, which supported the location of the acetate group.

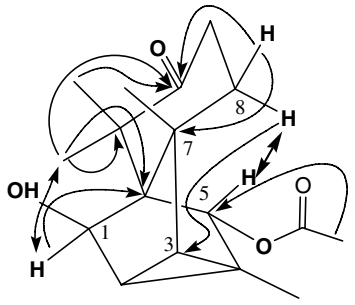


Fig. 3. Important NOE (double arrow) and HMBC correlations observed in **2**.

substantiated by the COSY correlation between H-1 and H-2, and H-2 and H-3. From the above data, the structure of **2** was deduced to be 5-acetoxy-1-hydroxycyclomyltaylan-10-one. The orientation of the acetate group at C-5 and the hydroxyl group at C-1 was confirmed by the observation of NOE correlations between H-5 and H-8a. The observation of the cross-peak between H-1 and H-15, together with the lack of correlations between H-1 and Me-13 and Me-14 in the NOESY spectrum indicated that the hydroxyl group at C-1 was pseudoequatorially oriented in the boat ring. The absolute configuration of **2** was deduced by comparison of its CD spectral data and its optical rotation with those of **1** ($[\alpha]_D^{17} - 23.2$). The negative value observed in **2** ($[\alpha]_D^{17} - 63.2$) and its negative Cotton effect at 292 nm, allowed us to determine the structure of **2** to be 5*R*-acetoxy-1*R*-hydroxycyclomyltaylan-10-one.

The positive HREIMS of compound **3** ($[\alpha]_D^{17} - 11.5$) gave a molecular formula of $C_{15}H_{22}O_3$, which differed from **2** by 42 mass units. The IR spectrum showed a characteristic absorption for hydroxyl groups (ν_{max} 3448 cm^{-1}). The ^1H and ^{13}C NMR spectroscopic data of **3** were very similar to those of **2** except for the absence of signals due to an acetate group, suggesting that **2** and **3** are the corresponding monoacetate and diol derivatives. Comparison of the ^{13}C NMR spectroscopic data of **2** with those of **3** revealed an upfield shift of the carbon resonance at C-5 (−0.9) and a downfield shift for the signals at C-6 (+0.3) and C-4 (+0.9) of **3** (Table 1), indicating that the acetoxyl group at C-5 in **2** was replaced by a hydroxyl group. The relative as well as absolute configurations of **3** were determined by NOESY analysis and comparison of its CD spectral data (negative Cotton effect at 295 nm) and optical rotation ($[\alpha]_D^{17} - 11.5$) with those of **1**. Thus **3** is 1*R*,5*R*-dihydroxycyclomyltaylan-10-one.

The positive HREIMS of compound **4** gave a molecular formula of $C_{19}H_{28}O_5$. Its IR spectrum showed the presence of two ester carbonyls (1740, 1720 cm^{-1}) and a hydroxyl group (3492 cm^{-1}). The ^1H NMR spectrum displayed signals for four quaternary methyl groups at δ: 0.81, 1.03, 1.14, and 1.34, two acetyl methyls (δ 2.13 and 2.16), and three oxygen-bearing methines at δ 4.16, (*td*, $J = 3.8$, 2.2 Hz, H-9), 5.00 (*s*, H-5), and 5.28 (*d*, $J = 3.8$, H-10). The ^{13}C NMR spectroscopic data for **4** showed 15 carbon signals assignable to a cyclomyltaylane sesquiterpene (Asakawa et al., 1991; Wei et al., 1995) and four signals due to two acetate groups (δ 21.0, 21.6, 170.4, and 170.5) attached to C-5 and C-10 (HMBC). Careful interpretation of the HMQC and ^1H–^1H-COSY spectra allowed us to conclude that **4** consists of two partial structures: –CH_2–CHO–CHO–, and –CH_2–CH–CH–. The HMBC spectrum showed correlations from H-10 to the two methyl groups at C-14 and C-15, from H-9 to C-7 and C-11, and from Me-12 to C-2, C-3, C-4, and C-5. The axial orientation of the hydroxyl group at C-9 and the equatorial orientation of that at C-10 were deduced from the above coupling constants and the observation of the NOE correlation (Fig. 4) between H-10 and H-8a (δ 2.40, *dd*, $J = 14.5$. 2.2 Hz). From the above data, the structure of **4** was established as 5*R*,10β-diacetoxycyclomyltaylan-9β-ol. Compound **4** is assumed to have the same absolute configuration as **1**–**3** since they are from the same liverwort sample.

The ^1H and ^{13}C NMR spectra of compound **5**, $C_{21}H_{30}O_7$ (HREIMS), were very similar to those of **4** except for the absence of a signal for a methyl group at C-13 and the presence of an acetylated hydroxymethylene moiety. On comparison of the ^{13}C NMR data of **4** with those of **5**, the chemical shifts of the signals due to C-7 were shifted downfield (+3.6 ppm), while those of C-3 and C-8 were shifted upfield (−5.1 and −5.8 ppm respectively), due to hydroxylation at C-13. Furthermore, HMBC correlations were observed between the hydroxymethylene at δ 3.95 and C-3, C-7 and C-8. The axial orientation of the hydroxyl group at C-9, and the equatorial orientation of that of C-10 were deduced from the coupling value observed at H-9 (δ 4.16, *td*, $J = 3.8$, 3.8 Hz) and H-10 (δ 5.29, *d*, $J = 3.8$ Hz), and the observation of a NOE cross-peak between H-10 and H-8a. Thus compound **5** is 5*R*,10β,13-triacetoxycyclomyltaylan-9β-ol.

Compound **6**, $C_{21}H_{30}O_7$ (HREIMS), had ^1H NMR signals which are very similar to those of **5** except for chemical shifts of H-9 and H-10. It was apparent that the acetate group was attached to C-9 (5.24, *td*, H-9), while C-10 (3.88, *d*, H-10) had a hydroxyl group. Interpretation of the NOE spectrum led to the assignment of compound **6** is 5*R*,9β,13-triacetoxycyclomyltaylan-10β-ol.

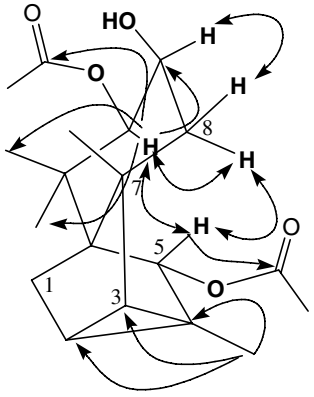


Fig. 4. Important NOE (double arrow) and HMBC correlations observed in **4**.

The absolute configurations of compounds **5** and **6** are suggested to be the same as **1**–**3** since they are originated from the same liverwort sample.

Compounds **1**–**6** were tested for inhibition of NO production in culture media on RAW 264.7 cells in response to lipopolysaccharide (LPS). However, none of the six cyclomyltaylanoids isolated showed any positive effects at 60 μM.

3. Concluding remarks

Cyclomyltaylanes are sesquiterpenoids found only in liverworts from the genera *Mylia*, *Bazzania*, *Reboulia*, and *Mannia* (Asakawa, 1995, 2004; Wei et al., 1995). They are suggested to be biosynthetically derived from β-chamigrene followed by migration of the C-3 methyl group to the vicinal C-4 (Takaoka et al., 1988; Harinantenaina et al., 2005). It is interesting that *B. madagassa*, *B. japonica*, and *B. tridens* all produce cyclomyltaylanoids, while myltayl-4(12)-en-5-ol (**7**) was only detected in *B. trilobata* (Nagashima et al., 1996; Asakawa, 1995). The latter is quite different from the other species since it also furnished lignans and chlorinated bisbibenzyls (Martini et al., 1998a,b).

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-1000 polarimeter with MeOH as 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 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). Preparative HPLC 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. RP-18 F_{254S} (20 × 20 cm) was used for preparative TLC. 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.

4.2. Plant material

B. madagassa was collected in Moramanga/Lakato (Madagascar) in June 2003 and identified by Prof. Emeritus T. Pocs of the Hungarian Academy of Sciences. A voucher specimen (2003LIV3) has been deposited in the Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

4.3. Extraction and isolation

Powdered *B. madagassa* (98 g) was extracted with Et_2O (500 ml) at room temperature for one week. The extract was filtered and concentrated *in vacuo* to yield a green oil (3 g), which was divided into eight fractions by column chromatography on Sephadex LH-20. Fraction 4 was subjected to silica gel CC (hexane:EtOAc, 4:1 to 100% EtOAc) to give compound **1** (16.9 mg). Silica gel CC (hexane:EtOAc 9:1 to 6:4) of the fifth fraction gave 16 sub-fractions. Fraction 5 (0.5 g) was applied to a silica gel column using hexane and ETOAc (4:1 to 100% EtOAc) as solvent to afford sixteen subfractions (5-1 to 5-16). (+)-Globulol (44.7 mg) was obtained from sub-fraction 5-6. The sub-fractions 5-10, 5-13 and 5-16 were applied to a ODS column eluted with MeOH:H_2O (7:3) to afford compounds **4** (3.5 mg), **2** (0.5 mg) and **3** (1.3 mg), as well as *ent*-4β,10α-dihydroxyaromadendrane (6.6 mg), respectively. Fraction 5-15 was purified by using ODS HPLC (MeOH–H_2O, 7:3) to yield compounds **5** and **6** (1.3 and 2.4 mg, respectively).

4.4. X-ray crystallographic analysis of *1*

Crystal data: colorless, needles; $C_{19}H_{26}O_5$, $M_r = 334.412$, orthorhombic, $P2_12_12_1$, $a = 7.9640(4)$ Å, $b = 14.3000(9)$ Å, $c = 16.586(2)$ Å, $\alpha = 90.00°$, $\beta = 90.00°$, $\gamma = 90.00°$, $V = 1888.9(2)$ Å^3, Mo Kα radiation, $\lambda = 0.71073$ Å, 1844 reflections, 217 parameters; only coordinates of H atoms refined, $R(gt) = 0.0661$, $wR(gt) = 0.1844$, $S(ref) = 1.081$. Data collection: DIP Image plate. Cell refinement: Scalepack (HKL), data reduction: maXus (Mackay et al., 1999). Program used to refine structure: SHELXL-97 (Sheldrick, 1997); refinement on F^2, full-matrix least-squares calculations. The atomic coordinates and equivalent isotropic displacement parameters, as well as a full list of bond distances and angles, and the structure factor table are deposited as supplementary material at the Cambridge crystallographic Data Centre (Deposition No. CCDC 270420). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033 or e-mail: deposit@ccdc.cam.ac.uk).

4.5. 1R,5R-diacetoxycyclomyltaylan-10-one (*1*)

Colorless needles (m.p. = 171–173°, $[\alpha]_D^{20} - 45.2$ (*c* 0.9, MeOH); CD: $\Delta\varepsilon_{298} - 12$ (MeOH, *c* 0.2); IR (KBr) cm^{-1}: 2917, 1739, 1709; Positive HREIMS: *m*/*z* 334.1784 $[M]^+$ ($C_{19}H_{26}O_5$, requires 334.1780).

4.6. 5R-acetoxy-1R-hydroxycyclomyltaylan-10-one (2)

Amorphous powder, $[\alpha]_D^{20} - 63.2$ (*c* 0.04, $CHCl_3$); CD: $\Delta\varepsilon_{292} - 14$ (MeOH, *c* 0.1) (IR (KBr): 3507, 2932, 1732, 1690, 1462, 1085 cm^{-1}. For ^1H NMR and ^{13}C NMR spectra, see Tables 1 and 2, respectively. Positive HREIMS *m/z* 292.1672 ($C_{17}H_{24}O_4$, requires *m/z* 292.1669).

4.7. 1R,5R-dihydroxycyclomyltaylan-10-one (3)

Amorphous powder, $[\alpha]_D^{17} - 11.5$ (*c* 1.3 $CHCl_3$); CD: $\Delta\varepsilon_{295} - 10$ (MeOH, *c* 0.5); IR (KBr): 3448, 2929, 1698, 1082 cm^{-1}. For ^1H NMR and ^{13}C NMR spectra, see Tables 1 and 2, respectively. Positive HREIMS *m/z* 250.1553 ($C_{15}H_{22}O_3$, requires *m/z* 250.1534).

4.8. 5R,10β-diacetoxycyclomyltaylan-9β-ol (4)

Amorphous powder, $[\alpha]_D^{17} + 241$ (*c* 0.08, $CHCl_3$); IR (KBr): 3492, 2962, 1740, 1720, 1451, 1051 cm^{-1}. For ^1H NMR and ^{13}C NMR spectra, see Tables 1 and 2, respectively. Positive HREIMS *m/z* 336.1928 ($C_{19}H_{28}O_5$, requires *m/z* 336.1920).

4.9. 5R,10β,13-triacetoxycyclomyltaylan-9β-ol (5)

Amorphous powder, $[\alpha]_D^{17} + 45.3$ (*c* 0.1, $CHCl_3$); IR (KBr): 3517, 2958, 1738, 1366, 1028 cm^{-1}. For ^1H NMR and ^{13}C NMR spectra, see Tables 1 and 2, respectively. Positive HREIMS *m/z* 394.1990 ($C_{21}H_{30}O_7$, requires *m/z* 394.1989).

4.10. 5R,9β,13-triacetoxycyclomyltaylan-10β-ol (6)

Amorphous powder, $[\alpha]_D^{17} + 67.2$ (*c* 0.2, $CHCl_3$); IR (KBr): 3453, 2957, 1736, 1448, 1038 cm^{-1}. For ^1H NMR and ^{13}C NMR spectra, see Tables 1 and 2, respectively. Positive HREIMS *m/z* 394.1990 ($C_{21}H_{30}O_7$, requires *m/z* 394.1989).

4.11. 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. Compounds **1**–**6** (60 μM) were 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 compounds **1**–**6** at 60 μM and stimulated with LPS at 4 μg/ml for 24 h.

4.12. 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 (35 μl) of Griess reagent (Quang et al., 2006), 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 each sample was calculated by means of the $NaNO_2$ serial dilution standard curve freshly prepared. Compounds **1**–**6** did not inhibit NO production of LPS-induced macrophage cells.

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