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ANTIALLERGIC DIMERIC PRENYLBENZOQUINONES FROM *EHRETIA MICROPHYLLA*

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Key Word Index—*Ehretia microphylla*; Boraginaceae; microphyllone; dehydromicrophyllone; hydroxymicrophyllone; cyclomicrophyllone; allomicrophyllone; exocytosis inhibitor; antigen-stimulated basophil.

Abstract—The ethyl acetate-soluble portion of the MeOH extract of *Ehretia microphylla* showed inhibitory activity on exocytosis in antigen-stimulated rat basophils. The bioassay-guided separation of this fraction afforded five biologically active compounds. By means of chemical and spectroscopic methods, the structures of these compounds, which include microphyllone, a unique dimeric prenylbenzoquinone, and its congeners, were elucidated.

INTRODUCTION

Ehretia microphylla Lamk. (syn. *Carmona retusa* (Vahl.) Masam.) is one of the most important medicinal plants in the Philippines, and is widely used for a variety of medicinal purposes. The decoction of the leaves is used as cure for coughs, and is prescribed for the treatment of diarrhoea with bloody discharge and for dysentery [1]. From the aerial part of this plant, a unique quinonoid compound, microphyllone (**1**), and two triterpenes, bauerenol and ursolic acid, have been isolated and characterized [2]. However, no bioassay-guided study has been carried out. In our previous study on the bioactive principles of the leaves of this plant, rosmarinic acid, which is an inhibitor of histamine release, and two flavonoid glycosides, astragalin and nicotoflorin, were isolated [3]. The present paper deals with the isolation of dimeric prenylbenzoquinones from the aerial part of this plant. The bioassay system used previously was modified for this study.

RESULTS AND DISCUSSION

The MeOH extract of the dried aerial part of *E. microphylla* was dissolved in water, then partitioned against *n*-hexane, EtOAc and *n*-BuOH. Each fraction was bioassayed for inhibitory activity on exocytosis of rat basophils caused by antigen-induced stimulation [4]. Significant activity was observed in the EtOAc fraction, which was further fractionated by silica gel column chromatography. From the most active fraction, five compounds (**1**–**5**) were isolated by ODS-HPLC.

Compound **1** was assigned the molecular formula $C_{22}H_{22}O_4$. The ^{13}C and ^1H NMR spectra of **1** showed the presence of two ketone groups, two trisubstituted double bonds, a 1,2,3,4-tetrasubstituted benzene, a *cis*-disubstituted double bond, three methyl protons with long-range coupling, two methylenes, a methine and two quaternary carbons (Table 1). These data suggested it to be a unique quinonoid compound, microphyllone (**1**), previously isolated from the same plant, and the structure of which was determined by X-ray crystallography of this dimethyl ether [2]. With the aid of ^1H–^1H COSY, HSQC and HMBC NMR measurement, all of the carbon and proton signals (Table 2) were reasonably assigned to this structure. The connectivities of carbons and protons are shown in Table 3. It should be noted that in the HMBC spectrum, the H-7 (δ 3.91) signal correlated with as many as nine carbon signals, C-1, C-2, C-3, C-8, C-9, C-1′, C-2′, C-3′ and C-7′. Thus H-7 was one of the key atoms which established the locations of the A and B rings as well as the side chains. Compound **1** was confirmed to be microphyllone on the basis of its mass ^1H and ^{13}C NMR data. However, some ^1H signal assignments differ from the reported data which were taken at 270 MHz. The reported $[\alpha]_D$ value ($-20°$ at *c* 2.0 in $CHCl_3$) was different from our value, $+6°$ at $c = 1.0$ in $CHCl_3$. The melting points were also different. Direct comparison could not be made due to the unavailability of a sample from the previous authors [2].

Since compound **1**, as well as compounds **2**–**5** (*vide post*), consisted of dimeric prenylbenzoquinone moieties,

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Table 1. ^{13}C NMR data of microphyllone (1) and its congeners (2–5)

C	1 $CDCl_3$	2 $CDCl_3$	3 CD_3OD	4 CD_3OD	5 $CDCl_3$
1	143.1	143.0	144.7	144.8	144.8
2	137.8	137.5	138.2	140.5	127.5
3	130.3	129.3	132.2	127.8	114.2
4	145.1	145.3	146.7	146.9	149.8
5	115.3	115.5	115.5	115.4	114.7
6	117.1	117.3	117.3	117.3	117.4
7	41.1	43.0	44.5	40.2	38.9
8	121.8	121.7	122.6	123.1	122.4
9	133.4	133.6	134.1	134.1	131.6
10	32.6	33.0	33.7	37.0	36.0
11	22.6	22.6	22.7	22.7	22.5
1′	202.7	198.7	201.0*	215.2	195.7
2′	68.4	69.2	70.4	64.7	54.7
3′	59.8	60.3	61.2	66.2	80.5
4′	201.6	201.1	202.3*	210.0	193.0
5′	139.2	139.3	140.3†	44.7	139.2
6′	139.8	140.3	140.9†	46.3	138.4
7′	28.7	123.3	123.4	25.6	121.8
8′	118.3	141.0	144.1	40.2	143.2
9′	135.3	142.0	71.2	143.5	70.9
10′	17.8	118.4	29.7	113.1	29.6
11′	25.8	18.2	29.9	23.8	29.6

*†Interchangeable values.

the numbering system adopted in the literature [2] was changed to correlate with the monomer unit. It reflects the biogenetic relationship between the congeners. In the following discussion, the hydroquinone ring and its prenyl side chain is termed the A-moiety (C-1 to C-11), and the benzoquinone ring with the prenyl unit is referred to as the B-moiety (C-1′ to C-11′).

Compound **2** was assigned a molecular formula, $C_{22}H_{20}O_4$ (FAB-MS), which had two fewer hydrogens than that of **1**. The ^{13}C and ^1H NMR spectra of **2** showed close similarities with those of **1**. The carbon signals of the A-moiety of **2** showed almost the same chemical shifts as those of **1**, except for the signal for C-7 which was shifted downfield by 1.9 ppm. In the case of the carbon signals of the B-moiety, those of the side chain were largely different. Two more double bond carbon signals appeared in place of the methylene and methyl carbons observed in **1**. The quinone carbonyl signal assigned to C-1′ and the signal assigned to C-2′ were shifted by -4 and $+0.8$ ppm, respectively. Taking the molecular formula into consideration, the structure of **2**, a 7′,9′-diene, was unequivocally assigned, and was named dehydromicrophyllone. The ^1H NMR spectrum of the B-side chain signals confirmed the structure (see Tables 1 and 2).

Compound **3** was assigned a molecular formula, $C_{22}H_{22}O_5$ (FAB-MS), which had one more oxygen than that of **1**. Comparison of the ^{13}C and ^1H NMR spectra of **3** with those of **1** revealed that the only difference observed was in the signals attributable to the side chain of the B-moiety. The structure of the B-side chain was readily proven to be a hydroxydimethylallyl group. Thus, the structure of **3** was as shown, and the compound was named hydroxymicrophyllone.

Compound **4** had the same molecular formula, $C_{22}H_{22}O_4$ (FAB-MS), as **1**. The ^{13}C and ^1H NMR spectra of **4** were compared with those of **1**. They suggested the presence of the same A-ring and the same A-side chain as those of **1**. In the case of the B-moiety, although two carbonyl carbons were still present, the double bond in the B-ring had disappeared, and two new signals, δ44.7 (*t*) and 46.3 (*d*), appeared instead (Table 1). The ^1H NMR signals of a $-CH_2-CH-$ system were also observed (Table 2). The side chain signals of the B-moiety were shifted and this suggested the presence of an isopropenyl group attached to a $-CH-CH_2$ system. By means of ^1H–^1H COSY, ^{13}C–^1H COSY and HMBC spectra, direct and long-range correlations of all carbons and skeleton protons were obtained (Table 4). It was found that H-6′ correlated not only with C-1′, C-2′, C-4′ and C-5′ but also with C-7′ of the B-side chain. And H-8′ correlated not only with C-7′, C-9′ and C-10′ but also with the C-5′ and C-6′ of the B-ring. This evidence was enough to prove that the B-side chain of **4** was cyclized with C-6′ of the B-ring to make a new five-membered ring. All of the other correlated carbons and protons confirmed the cyclic structure at the C-6′ and C-8′ position of the microphyllone, to which we gave the name cyclomicrophyllone.

Compound **5** was assigned a molecular formula, $C_{22}H_{22}O_5$ (FAB-MS), which had one more oxygen atom than **1**. The ^{13}C and ^1H NMR spectra of **5** were compared with those of **1**. All of the carbon atoms of **5** could be attributed to two prenyl benzoquinone moieties, A and B, as in the case of **1**. However, the significant difference between these compounds is that **5** had a 1,2,4-substituted benzene ring, while **1** had a 1,2,3,4-substituted ring. By means of ^1H–^1H COSY, HSQC and HMBC spectra, direct and long-range correlation, all carbons and skeleton protons were assigned (Table 5). As for **1**, H-7 correlated with nine carbons in the A and B-rings, and A- and B-side chains, and the H-3 signal (δ6.50, *d*, $J = 3.0$ Hz) correlated with C-1, C-5 and C-7. This proved that C-7 was the key atom that connects the A- and B-rings. The structure of the B-side chain was readily attributed to a hydroxy dimethylallyl group by comparison of the NMR spectra with those of **3**. The C-3′ signal was shifted downfield by 19.3 ppm, and this could reasonably be explained only by C–O bond formation on C-3′. The position of the bonded oxygen was unequivocally decided to be C-1–O, since a NOE was observed between H-3 and H-7. Thus the structure of **5** was as shown, and the compound was named allomicrophyllone.

The biogenesis of these five compounds probably starts from two prenylbenzoquinone molecules. A Diels–Alder type reaction between the prenylbenzoquinone (B) and its equivalent diene-hydroquinone (A) would give **6**, the common precursor of the five compounds. Oxidative coupling between C-3 and C-3′ of

Table 2. ^{1}H NMR data of microphyllone congeners, in deuterated chloroform (**1**, **2** and **5**) or deuterated methanol (**3** and **4**)

H	Microphyllone (**1**)	Dehydromicrophyllone (**2**)	Hydroxymicrophyllone (**3**)	Cyclomicrophyllone (**4**)	Allomicrophyllone (**5**)
1	5.60 (OH, *br s*)				
3					6.50 (*d*, *J* = 3.0)
4	5.53 (OH, *br s*)				
5	6.34 (*d*, *J* = 8.6)	6.39 (*d*, *J* = 8.5)	6.33 (*d*, *J* = 8.8)	6.32 (*d*, *J* = 8.5)	6.45 (*dd*, *J* = 3.0, 8.6)
6	6.47 (*d*, *J* = 8.6)	6.52 (*d*, *J* = 8.5)	6.42 (*d*, *J* = 8.8)	6.45 (*d*, *J* = 8.5)	6.54 (*d*, *J* = 8.6)
7	3.91 (*d*, *J* = 6.7)	4.12 (*d*, *J* = 6.8)	4.01 (*d*, *J* = 6.6)	3.46 (*d*, *J* = 6.6)	3.70 (*d*, *J* = 6.3)
8	5.81 (*dq*, *J* = 6.7, 1.2)	5.83 (*dq*, *J* = 6.8, 1.5)	5.82 (*dq*, *J* = 6.6, 1.2)	5.81 (*dq*, *J* = 6.6, 1.5)	5.57 (*dq*, *J* = 6.3, 1.0, 1.0)
10	2.28 (*d*, *J* = 18.8)	2.33 (*d*, *J* = 18.6)	2.13 (*d*, *J* = 18.3)	2.28 (*d*, *J* = 19.0)	2.43 (*d*, *J* = 19.3)
	2.68 (*d*, *J* = 18.8)	2.67 (*d*, *J* = 18.6)	2.63 (*d*, *J* = 18.3)	2.65 (*d*, *J* = 19.0)	2.66 (*br d*, *J* = 19.3)
11	1.61 (3H, *d*, *J* = 1.2)	1.56 (3H, *br s*)	1.56 (3H, *d*, *J* = 1.2)	1.58 (3H, *d*, *J* = 1.5)	1.62 (3H, *br s*)
5′	6.73 (*d*, *J* = 10.2)	6.78 (*d*, *J* = 10.5)	6.77 (*d*, *J* = 10.3)	2.63 (*dd*, *J* = 2.9, 17.1)	6.76 (*d*, *J* = 10.5)
				2.84 (*dd*, *J* = 3.9, 17.1)	
6′	6.43 (*d*, *J* = 10.2)	6.53 (*d*, *J* = 10.5)	6.42 (*d*, *J* = 10.3)	2.58 (*ddd*, *J* = 2.9, 3.9, 6.8)	6.43 (*d*, *J* = 10.5)
7′	2.31 (*dd*, *J* = 7.8, 14.8)	5.60 (*d*, *J* = 16.6)	5.65 (*d*, *J* = 16.2)	1.91 (*dd*, *J* = 11.5, 14.6)	5.60 (*d*, *J* = 16.1)
	2.47 (*dd*, *J* = 7.8, 14.8)			2.20 (*dd*, *J* = 5.9, 14.6)	
8′	4.95 (*tsept*, *J* = 7.8, 1.0)	6.20 (*d*, *J* = 16.6)	5.70 (*d*, *J* = 16.2)	2.90 (*ddd*, *J* = 5.9, 6.8, 11.5)	5.69 (*d*, *J* = 16.1)
10′	14.7 (3H, *d*, *J* = 1.0)	4.95 (*br s*)	1.16 (3H, *s*)	4.88 (*s*)	1.15 (3*H*, *s*)
		5.00 (*br s*)		5.11 (*s*)	
11′	1.64 (3H, *d*, *J* = 1.0)	1.75 (3H, *dd*, *J* = 0.5, 0.7)	1.16 (3H, *s*)	1.76 (3H, *s*)	1.15 (3H, *s*)

^{1}H signals unless otherwise indicated (*J* values in Hz).

Table 3. HMBC (*) and direct C–H (○) correlation of microphyllone (**1**)

	C	A-ring						A-side chain					B-ring						B-side chain				
H		1	2	3	4	5	6	7	8	9	10	11	1′	2′	3′	4′	5′	6′	7′	8′	9′	10′	11′
A-ring	5	*		*	*	○																	
	6	*	*		*		○																
A-side chain	7	*	*	*				○	*	*			*	*	*				*				
	8							*	○		*	*		*									
	10a			*					*	*	○			*	*								
	10b			*					*	*	○				*								
	11								*	*	*	○											
B-ring	5′												*		*		○						
	6′													*		*		○					
B-side chain	7′a												*	*	*				○	*	*		
	7′b												*	*	*				○	*	*		
	8′																			○		*	*
	10′																			*	*	○	*
	11′																			*	*	*	○

Table 4. HMBC (*) and direct C–H (○) correlation of cyclomicrophyllone (**4**)

H	C	A-ring							A-side chain				B-ring							B-side chain			
		1	2	3	4	5	6	7	8	9	10	11	1′	2′	3′	4′	5′	6′	7′	8′	9′	10′	11′
A-ring	5	*		*	*	○																	
	6	*	*		*		○																
A-side chain	7	*	*	*				○	*	*			*	*	*								
	8								○		*	*		*									
	10a			*					*	*	○	*		*	*								
	10b			*					*	*	○				*								
	11								*	*	*	○											
B-ring	5′a																○	*					
	5′b																○						
	6′												*	*		*	*	○	*				
B-side chain	7′a							*						*	*			*	○	*			
	7′b							*					*	*	*				○	*	*		
	8′																*	*	*	○	*	*	
	10′a																			*	*	○	*
	10′b																			*		○	*
	11′																				*	*	○

Table 5. HMBC (*) and direct C–H (○) correlation of allomicrophyllone (**5**)

H	C	A-ring							A-side chain				B-ring							B-side chain			
		1	2	3	4	5	6	7	8	9	10	11	1′	2′	3′	4′	5′	6′	7′	8′	9′	10′	11′
A-ring	3	*		○		*		*															
	5	*		*		○																	
	6	*	*		*		○																
A-side chain	7	*	*	*				○	*	*			*	*	*				*				
	8								○		*	*		*									
	10a								*	*	○	*		*	*								
	10b								*	*	○												
	11								*	*	*	○											
B-ring	5′												*		*		○						
	6′													*		*		○					
B-side chain	7′							*					*		*				○	*	*		
	8′													*					*	○	*	*	*
	10′																			*	*	○	
	11′																			*	*		○

6 (route a) would yield **1**, while coupling between C-1–O and C-3′ (route b) would yield **5**. Compounds **2** and **3** could be derived from **1** by oxidation. Cyclization of **2** between C-6′ and C-8′ would give **4** (Fig. 1).

Each compound was bioassayed for its inhibitory activity on exocytosis of rat basophils caused by antigen-induced stimulation. Among the five compounds, **1** and **5** showed strong activity (IC_{50} = 33 and 36 μM, respectively) while **2** and **4** had weaker activity (IC_{50} = 62 and 106 μM, respectively) and **3** was inactive. The data suggest that the quinonoid structure of the B-ring may be responsible for some of the activity. However, simple addition of activities based on the partial structures does not constitute the total activity, partly because of the steric relationships of each partial structure.

EXPERIMENTAL

Mps: uncorr.; ^{1}H NMR and ^{13}C NMR (TMS as int. standard): 400 and 100 MHz, respectively; FAB-MS: negative mode.

Measurement of hexosaminidase release. For measurement of the secretion of hexosaminidase, RBL-2H3 cultures were incubated with DNP-specific IgE_4 (Sigma) in complete growth medium in 96-well plates (4×10^4 cells/80 μl of medium/well) [4]. Cultures were washed twice, and the medium was replaced with a glucose–saline PIPES-buffered medium that contained 1 mM Ca^{2+} (buffer A). Cells were pre-incubated for 10 min at 37° in 40 μl buffer A, containing the MeOH extract of *Ehretia microphylla* or isolated compounds which were prepd in DMSO and diluted to give < 0.1% DMSO solns [5]. The cells were then stimulated with DNP–BSA (20 ng ml^{-1}) for 15 min. An aliquot (10 μl) of the medium and cell lysate (in 50 μl 0.1% Triton X-100) was incubated with 10 μl 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glycosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37° for 1 hr. At the end of the incubation, 250 μl 0.1 M Na_2CO_3–0.1 M $NaHCO_3$ buffer (pH 10) was added, and the absorption of the solution measured at 410 nm. Values (mean ± S.E.) were expressed as the actual release (percent of total hexosaminidase) after correction for spontaneous release (2–3%) or as a per cent of maximal response.

Plant material. Ehretia microphylla was collected in Yomitanson, Okinawa, Japan in August 1989. A voucher specimen is deposited in the Herbarium of the Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine (Bor-8901).

Extraction and isolation. The sliced stem (fr. wt 2.0 kg) was extracted with hot MeOH to give 151 g of extract, a part (76 g) of which was dissolved in H_2O and extracted with hexane, EtOAc, and *n*-BuOH, successively, to give 7.4 g, 11 g, 3.3 g of the respective extracts, and remaining aq. portion (46 g). The bioactivity of each of these frs. was measured at a concn of 1 mg ml^{-1} soln and found to be 50, 95, 25 and 0%, respectively. The portion (5 g) of the most active fr. (EtOAc) was chromatographed on a column of silica gel using hexane–EtOAc (2:1 to 3:2), EtOAc, EtOAc–MeOH (2:1) and MeOH to give 8 frs

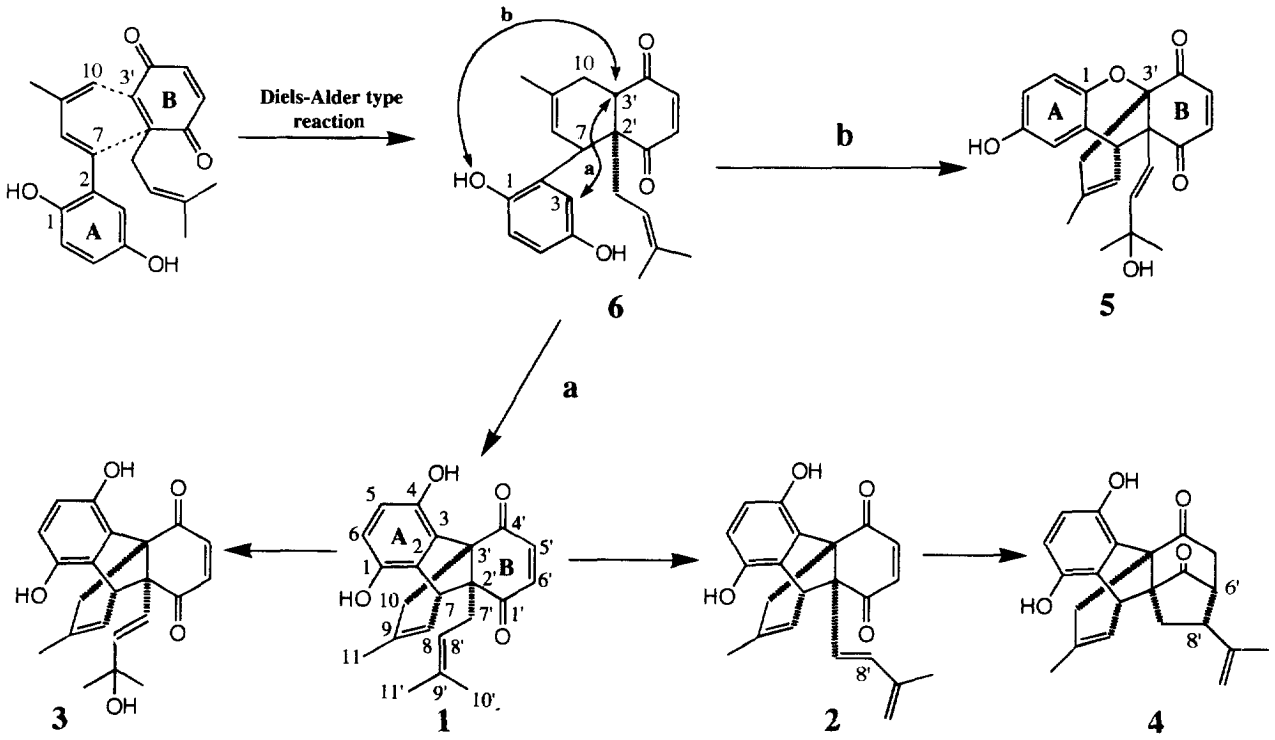

Fig. 1. Probable biogenesis of microphyllone (**1**) and its congeners (**2**–**5**).

(E-1 to E-8). The weights (mg) and activities (%) of E-1 to E-8 at 1 mg ml^{-1} were: 120 (92), 490 (95), 91 (0), 85 (0), 68 (0), 670 (60), 2.7 g (56) and 690 (57), respectively. From E-6 and E-7, rosmarinic acid (49 mg) was obtained. The most active fr., E-2, was further chromatographed on silica gel using CH_2Cl_2–MeOH (50:1 to 20:1) and MeOH to give 6 frs of the following weights and activities at 1 mg ml^{-1}; 150 mg (0%), 7 mg (50%), 280 mg (100%), 21 mg (0%), 28 mg (30%) and 37 mg (30%). The strongest (third) of these frs (96% at 100 μg ml^{-1}) was fractionated on HPLC (ODS) using MeOH–H_2O (13:7 and 7:13) and MeCN–H_2O (2:3) to yield compounds **3** (1 mg), **4** (2 mg), **5** (3 mg), **2** (6 mg) and **1** (140 mg) in order of elution.

Microphyllone (**1**). Pale yellow crystals from C_6H_6. Mp 167.5–168° (lit. 179–180° from C_6H_6 [2]). $[\alpha]_D^{19}$ + 6.0° ($CHCl_3$; *c* 1.0) (lit. −20.0°, $CHCl_3$; *c* 2.0 [2]); CD (MeOH; *c* 0.09): $[\theta]_{235} + 8.2 \times 10^4$; FAB-MS *m/z*: 350 $[M]^-$; ^{13}C NMR: Table 1; ^{1}H NMR: Table 2. Data essentially identical with ref. [2].

Dehydromicrophyllone (**2**). Pale yellow powder. $[\alpha]_D^{19}$ −58° ($CHCl_3$; *c* 0.53); CD (MeOH; *c* 0.08): $[\theta]_{236} + 3.6 \times 10^4$; HR-FAB-MS *m/z*: 348.1371, $[M]^-$ $C_{22}H_{20}O_4$ requires 348.1362; ^{13}C NMR: Table 1; ^{1}H NMR: Table 2.

Hydroxymicrophyllone (**3**). Pale yellow powder. $[\alpha]_D^{19}$ + 7.5° (MeOH; *c* 0.28); CD (MeOH; *c* 0.07): $[\theta]_{225} + 6.5 \times 10^4$; HR-FAB-MS *m/z* 366.1467 $[M]^-$, $C_{22}H_{22}O_5$ requires 366.1467; ^{13}C NMR: Table 1; ^{1}H NMR: Table 2.

Cyclomicrophyllone (**4**). Pale yellow powder. $[\alpha]_D^{19}$ + 85.7° (MeOH; *c* 0.23); CD (MeOH; *c* 0.07): $[\theta]_{226} - 2.7 \times 10^4$, $[\theta]_{259} + 4.1 \times 10^4$, $[\theta]_{293} - 4.9 \times 10^4$, $[\theta]_{317} + 2.0 \times 10^4$; HR-FAB-MS *m/z*: 349.1427 $[M-H]$, $C_{22}H_{21}O_4$ requires 349.1440; ^{13}C NMR: Table 1; ^{1}H NMR: Table 2.

Allomicrophyllone (**5**). Pale yellow powder. $[\alpha]_D^{19}$ + 25° ($CHCl_3$; *c* 0.12); CD (MeOH; *c* 0.08): $[\theta]_{219} - 2.5 \times 10^4$, $[\theta]_{240} + 1.8 \times 10^4$; HR-FAB-MS *m/z*: 366.1434 $[M]^-$, $C_{22}H_{22}O_5$ requires 366.1467; ^{13}C NMR: Table 1; ^{1}H NMR: Table 2.

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