Inhibitory Effect of Macabarterin, a Polyoxygenated Ellagitannin from *Macaranga barteri*, on Human Neutrophil Respiratory Burst Activity

Rostand Manfouo Ngoumfo,*[,†] Georges Eric Ngounou,[†] Chimene Victoire Tchamadeu,[†] Muhammad Irfan Qadir,[‡] Celine Djama Mbazoa,[†] Afshan Begum,[‡] Fernande Ngounou Ngninzeko,[†] David Lontsi,*[,†] and Muhammad Iqbal Choudhary[‡]

Department of Organic Chemistry, Faculty of Science, University of Yaounde I, P.O. Box 812, Yaounde, Cameroon, and HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

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An ellagitannin with a 2,4-acyl group, named macabarterin (**1**), and a new ellagic acid glycoside, 3-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (**2**), were isolated from the stem bark extract of *Macaranga barteri* along with five known phenolic compounds, ellagic acid (**3**), 3-*O*-methylellagic acid (**4**), gallic acid (**5**), methyl gallate (**6**), and scopoletin (**7**). The structures of **1** and **2**, as well as those of the known compounds, were elucidated on the basis of spectroscopic data and by comparison with reported data. Compounds **1**−**5** and **7** were tested for their anti-inflammatory potential in a cell-based respiratory burst assay, compound **1** being found an inhibitor of the superoxides produced in the cellular system.

Macaranga is a large genus of Old World tropical trees of the Euphorbiaceae family and the only genus in the subtribe Macaranginae. Native to Africa, Asia, and the South Pacific, the genus comprises over 300 species.[1] *Macaranga barteri* Muell. Arg. has not been investigated. Among more than 8000 known plant polyphenols, flavonoids, quinones, lignans, xanthones, and coumarins are the most abundant.[2] Some of these secondary metabolites, isolated from various species of the genus *Macaranga*, showed anti-inflammatory activity.[3,4] Inflammation is a defensive response that induces physiological adaptations to limit tissue damage and removes the pathogenic infections.[5] Bacterial infections cause an increased number of neutrophils, which plays a crucial role by killing and digesting pathogens. Reactive oxygen species (ROS) are formed subsequent to the assembly and activation of the phagocyte-specific enzyme NADPH oxidase. This process is initiated by the production of superoxide anion, during a "respiratory burst" of non-mitochondrial oxygen uptake by an NADPH oxidase system.[6,7]

Based on our interest in bioactive phenolic metabolites of the Euphorbiaceous plants, a study of the phenolic constituents of *M. barteri* was undertaken. In the present work, the MeOH extract of the stem bark of *M. barteri* yielded seven phenolic compounds, including a highly oxygenated ellagitannin and an ellagic acid glycoside, named macabarterin (**1**) and 3-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (**2**), respectively. The anti-inflammatory activity of compounds **1**−**5** and **7** was evaluated in an *in vitro* assay. The assay involves water-soluble tetrazolium salt (WST-1) to measure the superoxide production of neutrophils activated by opsonized zymosan, which induces phagocytic activation of neutrophils. This technique is more sensitive and reliable than other available methods.[8]

Results and Discussion

The air-dried, powdered stem bark of *M. barteri* was extracted with MeOH, and the extract was first fractionated by silica gel 60 (Fluka, 230−400 mesh) column chromatography. These fractions were subjected to repeated column chromatography over Sephadex LH-20 to yield 3-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (**2**), ellagic acid (**3**),[9] 3-*O*-methylellagic acid (**4**),[9] gallic acid (**5**),[10] methyl gallate (**6**),[11] and scopoletin (**7**).[12] Compound **1** was purified by recycling preparative HPLC. Analyses of 1D and 2D NMR and mass spectra were used for structure determination. The known compounds were identified by direct comparison of the spectroscopic and physical data with reported data.

Macabarterin (**1**) was obtained as a brown, amorphous powder and characterized as an ellagitannin by its positive reaction with ferric chloride reagent (dark blue coloration) and sodium nitrite-acetic acid (reddish-brown coloration).[13,14] Its HRESIMS showed an $[M + H]^+$ ion at *m/z* 1103.1621, corresponding to the formula $C_{46}H_{38}O_{32}$, with 28 degrees of unsaturation, among which 23 were accounted for by four aromatic rings, one sugar ring, two rings due to the 2,4-acyl group and 3,5-hexahydroxydiphenic acid (HHDP) substitutions of the β-D-glucopyranose, as found in many ellagitannins such as geraniin[9] and jolkinin,[10] and four aliphatic rings in the acyl group. The remaining five degrees of unsaturation indicated that there were five carbonyl ester groups clearly visible in the ^{13}C NMR spectrum at δ 166.2, 166.6, 167.5, 170.1, and 171.4 (see Table 1). The presence of the four aromatic rings and of the sugar ring in **1** was inferred from the ^1H NMR spectrum, which displayed four singlets of five protons at δ 7.31 (1H, H-1, acyl group), 7.08 (2H, H-2 and H-6, galloyl), 6.86 (1H, H-1, HHDP), and 6.62 (1H, H-1′, HHDP) with a board singlet at δ 6.50, assigned to the anomeric proton of the β-D-glucopyranose moiety. The ^1H and ^{13}C NMR data listed in Table 1, and the $^1H-^1H$ COSY, NOESY, HMQC, and HMBC experiments indicated the presence of the galloyl, HHDP, and acyl esters on the β-D-glucopyranose moiety.[15]

Following identification of the three main parts of **1**, it was observed that the uncommon part is the acyl group containing an aromatic ring (C-1 to C-6), two ester carbonyl (C-7 and C-7′), five aliphatic quaternary (C-2′, C4′, C-5′, C-6′, and C-2″), four methine (C-1′, C-3′, C-3″, and C-4″), and two methylene (C-1″ and C-5″) carbons. The 2,4-substitution of the β-D-glucopyranose was inferred from the HMBC cross-peaks between glucose proton signals at δ 5.25 (br s, H-4) and 5.49 (m, H-2) and carbonyl carbons C-7′ (δ 171.4) and C-7 (δ 166.6). Chemical shifts of aromatic carbons (Table 1) were characteristic of a trioxygenated ring. HMBC correlations of the singlet at δ 7.31 with carbons C-1, C-2, C-4, C-5, and C-7 indicated the presence of the ester carbonyl carbon attached at C-2. In the HMQC spectrum, the proton at δ 4.77 was correlated to the methine carbon at δ 52.5 (C-1′). H-1′ showed important HMBC correlations (Figure 1) with aromatic carbons at δ 111.9 (C-1), 119.7 (C-2), and 145.3 (C-6), a methylene carbon

* Corresponding authors. Tel: +237 77 62 51 66 (R.M.N.)/+237 77 76 27 80. Fax: +237 22 22 91 16 (D.L.) E-mail: monordr@yahoo.com (R.M.N.); dlontsi2000@yahoo.co.uk (D.L.).

† University of Yaounde I.

‡ University of Karachi.

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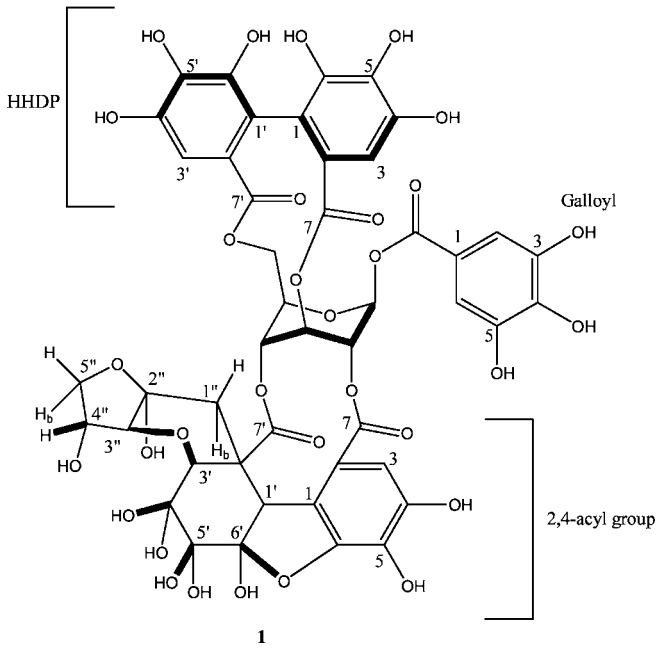

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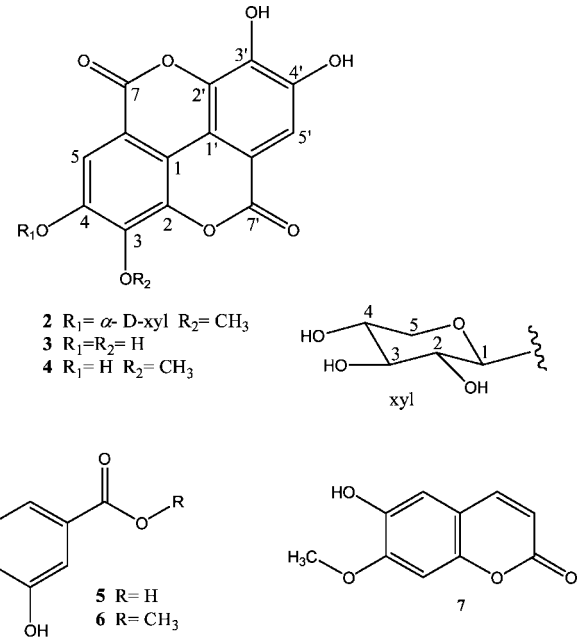

at δ 32.7 (C-1″), a carbonyl carbon at δ 171.4 (C-7′), and acetal carbons at δ 99.5 (C-5′) and 99.0 (C-6′). An additional methine proton observed at δ 4.99 (s, H-3′) in the ^{1}H NMR spectrum of **1** showed HMBC correlations with C-1″ (CH_2, δ 32.7), C-2′ (C, δ 54.0), C-3″ (CH, δ 82.1), C-5′ (C, δ 99.5), C-4′ (C, δ 99.4), and C-7′ (C, δ 171.4). The correlation of H-3′ with the ester carbonyl C-7′ indicated its location. Correlations of H-1′ and H-3′ suggested the presence of the cyclohexane ring. This was further confirmed in the ^{1}H−^{1}H COSY spectrum by the correlation between H-1′ and H-3′. These also indicated that C-7′ was attached to C-2′. The ^{1}H−^{1}H COSY spectrum of **1** showed correlations between oxymethine and oxy-methylene protons H-4″ and H-5″. The resulting HMBC correlations (Figure 1) with the acetal C-2″ (δ 110.2) and the methine C-3″ (δ 82.1) revealed the presence of the tetrahydrofuran ring formed by C-2″, C-3″, C-4″, and C-5″, as shown in Figure 1. The remaining tetrahydropyran ring was the consequence of the connection between the tetrahydrofuran and cyclohexane rings. This connection was justified by the chemical shifts of C-2′ (δ 54.0) and C-3′ (δ 77.7) together with the HMBC correlations of H-1″ and H-3′. The result of a 1D TOCSY mixing time experiment was very useful for the confirmation of this connection. In fact, separate irradiation of the two methylene protons (δ 1.56 and 2.80) revealed after 60 ms two singlets at δ 4.77 (H-1′) and 4.99 (H-3′). Also, the different chemical shift values of the geminal protons of C-1″ and C-5″ were indicative of their diastereotopicity. The relative spatial positions of the acyl group protons of **1** were determined, by using the key NOESY experiment (Figure 1). All these spectroscopic and physical data compared to the related ellagitannins, previously isolated from various Euphorbiaceous species,[13−16] led us to conclude that macabacterin (**1**) is a new polyoxygenated ellagitannin.

Compound **2** was obtained as a white, amorphous powder. The molecular ion $[M + H]^+$ at *m/z* 449.1165 observed in the HRESIMS and the ^{1}H, ^{13}C, and DEPT NMR data revealed the molecular formula $C_{20}H_{16}O_{12}$. Characteristic UV absorptions in DMSO suggested the presence of an ellagic acid moiety.[17] Acid hydrolysis of **2** afforded **4**, identified by direct comparison of TLC and its ^{1}H NMR spectrum. The ^{13}C NMR and DEPT spectra exhibited 12 quaternary carbons (Table 1) including 10 aromatic and two carbonyl carbons (δ 158.6 and 158.8), six carbons with two isolated aromatic (δ 110.3 and 111.8) and four oxygen-bearing carbons characteristic of a sugar moiety (δ 69.2, 73.0, 76.1, and 101.9), one oxy-methylene (δ 65.7), and one *O*-methyl carbon (δ 61.5). Furthermore, ^{1}H NMR, ^{1}H−^{1}H COSY, and HMQC data were in good agreement with those reported for ellagic acid glycosides.[9,17−19] The methoxyl group was placed at C-3 on the basis of the HMBC correlations of its protons (δ 4.06) with the aromatic carbon at δ 141.8, which also exhibited a cross-peak (^3J correlation) with H-5 at δ 7.74. A cross-peak between H-5 and C-4 at δ 151.4 (^2J correlation) was also observed. The anomeric proton at δ 5.12 (d, J = 7.2 Hz) showed a ^3J HMBC correlation with C-4 (δ 151.4), indicating that the sugar moiety was located at C-4. The coupling constant (J = 7.2 Hz) was in good agreement with the β-configuration. The sugar was finally identified to be β-D-xylose by comparison of its specific rotation, after acid hydrolysis of **2**, and comparison of ^{1}H and ^{13}C NMR chemical shifts with the literature data.[20,21] The three cross-peaks displayed by the anomeric proton (Figure 2) in the NOESY spectrum indicated the spatial interaction between three axial protons of the sugar moiety.[22] Other NOESY and HMBC correlations (Table 1) indicated that compound **2** is a new ellagic acid derivative, 3-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside.

Respiratory Burst Inhibitory Activity. The primary function of neutrophilic polymorphonuclear leukocytes (neutrophils) in the innate immune response is to control and kill invading microbial pathogens. This is achieved through a series of rapid and coordinated responses culminating in phagocytosis and killing of the pathogens. Neutrophils possess a membrane-bound multicomponent enzyme complex termed NADPH oxidase. When activated, it generates large quantities of ROS.[7,23,24] This system is responsible for the neutrophil "respiratory burst" (increased respiration of phagocytosis). Molecules that exhibit respiratory burst inhibitory activity may have potential as anti-inflammatory agents. Compounds **1**−**5** and **7** were evaluated by using this contemporary assay.[25] Indomethacin and aspirin, two clinically used anti-inflammatory drugs, were used as positive control. The concentration of compounds at which the superoxide production was suppressed up to 50% (IC_{50}) and the percentage inhibition of reduction of WST-1 compared with standards are summarized in Table 2. The polyoxygenated ellagitannin macabarterin (**1**) was found to be the most active compound in the series and identified as a potential nonsteroidal anti-inflammatory. The remaining compounds were largely inactive (IC_{50} > 1000 μg/mL).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP-360 digital polarimeter in MeOH solution; IR spectra were measured in KBr on a Perkin-Elmer 1600 FTIR spectrophotom-

Table 1. NMR Spectroscopic Data for Macabarterin (**1**) (400 MHz, CD_3OD) and 3-*O*-Methylellagic Acid 4-*O*-β-D-Xylopyranoside (**2**) (500 MHz, DMSO-d_6)[a]

	macabarteriin (**1**)				3-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (**2**)			
position	δ_C	mult.	δ_H (J in Hz)	HMBC	δ_C	mult.	δ_H (J in Hz)	HMBC
		HHDP				3-*O*-methylellagic acid group		
1	117.6	qC			114.4	qC		
2	124.5	qC			141.2	qC		
3	110.4	CH	6.86, s	$1,2_w,4_w,5,7$	141.8	qC		
4	145.4	qC			151.4	qC		
5	138.7	qC			111.8	CH	7.74, s	$1,3,4_w,6_w,7$
6	145.6	qC			112.2	qC		
7	167.5	qC			158.8	qC		
1′	116.3	qC			111.6	qC		
2′	125.5	qC			136.5	qC		
3′	108.2	CH	6.62, s	$1',2'_w,4'_w,5',7'$	140.2	qC		
4′	146.1	qC			148.9	qC		
5′	137.6	qC			110.3	CH	7.46, s	$1',3',4'_w,6'_w,7'$
6′	145.6	qC			107.5	qC		
7′	170.1	qC			158.6	qC		
3-OCH_3 sugar					61.5	CH_3	4.06, s	4
1	92.4	CH	6.50, br s	$2,3,5,7_G$	101.9	CH	5.12, d (7.2)	$2,3,4^b,5$
2	70.8	CH	5.49, m	$1,3,4,7_{AG}$	73.0	CH	3.33, m	
3	63.5	CH	5.54, m	$1,2,4,5,7_{HHDP}$	69.2	CH	3.41, m	
4	66.6	CH	5.25, m	2,3	76.1	CH	3.31, m	
5	73.9	CH	4.80, m	1,4	65.7	CH_2	3.38, br d (4.7)	1,3,4
							3.82, dd (4.7)	
6	64.7	CH_2	4.35, m	$4,5,7'_{HHDP}$				
			4.80, m					
galloyl								
1	120.1	qC						
2	110.9	CH	7.08, s	$1_w,3_w,4,6,7$				
3	146.6	qC						
4	140.9	qC						
5	146.6	qC						
6	110.9	CH	7.08, s	$1_w,2,4,5_w,7$				
7	166.2	qC						
acyl group								
1	111.9	qC						
2	119.7	qC						
3	115.3	CH	7.31, s	$1,2_w,4_w,5,7$				
4	146.5	qC						
5	139.3	qC						
6	145.3	qC						
7	166.6	qC						
1′	52.5	CH	4.77, s	$1,2,6,3'',5',6',7'$				
2′	54.0	qC						
3′	77.7	CH	4.99, s	$1'',4',3'',1',7'$				
4′	99.4	qC						
5′	99.5	qC						
6′	99.0	qC						
7′	171.4	qC						
1″	32.7	CH_2	1.56, d (14)	$1',2',3',3'',7'$				
			2.80[c], d (14)					
2″	110.2	qC						
3″	82.1	CH	4.17, br s	4″,5″				
4″	78.0	CH	4.13, m	2″,3″,5″				
5″	75.6	CH_2	3.92, m	2″,3″,4″				
			4.22[c], br d (8)					

[a] w: weak; G: galloyl; AG: acyl group. [b] Ellagic acid carbon. [c] See Figure 1.

eter. ^1H NMR, ^{13}C NMR, $^1H-^1H$ COSY, NOESY, TOCSY, HMQC, and HMBC spectra were recorded in CD_3OD and DMSO-d_6 on a Varian VXR-500 spectrometer operating at 400 and 500 MHz for ^1H and 100 and 125 MHz for ^{13}C of **1** and **2**, respectively, with TMS as internal reference. HRESIMS spectra were recorded by using a QSTAR XL mass spectrometer (LC/MS/TOF system). Open column liquid chromatography was performed over silica gel (Merck, 60−200 mesh) and Sephadex LH-20. Recycling preparative HPLC (RPHPLC) separation was performed on a JAI (Japan Analytical Industry) LC-908W with column of YMC ODS L-80. Analytical TLC was performed on precoated Merck glass sheets (Whatman K_6F silica gel 60 Å), viewed under a UV lamp and sprayed with $Ce_2(SO_4)_3$ solution (1 g/100 mL of distilled H_2O) in 10% H_2SO_4 and 2% ethanolic $FeCl_3$ for tannins. During the biological tests, absorbance was measured on a SpectraMax 340 microplate reader (Molecular Devices CA). WST-1 (Dojindo Laboratories, Kumamoto, Japan) and zymozan A (Sigma Chemicals, St Louis, MO) were used. The water was redistilled and deionized.

Plant Material. Stem barks of *Macaranga barteri* Muell. Arg. were collected from Mont Kalla, Center Province of Cameroon, in November 2007. The plant was identified by M. Nana, Botanist at the National Herbarium of Cameroon, where a voucher specimen (No. 6703/SFR/CAM) was deposited.

Extraction and Isolation. The air-dried powdered stem barks (1.2 kg) were extracted with MeOH to furnish 78 g of extract. A part (60 g) of this extract was subjected to an open chromatographic column using silica gel as adsorbent. Elution was performed with *n*-hexane, *n*-hexane−EtOAc, and EtOAc−MeOH in increasing polarity. Subfractions (198 × 500 mL) were collected and grouped in 14 fractions (F_1-F_{14}) on the basis of TLC. Fractions F_5 (50/50 *n*-hexane−EtOAc) and F_8 (EtOAc) were further separated by repeated Sephadex LH-20

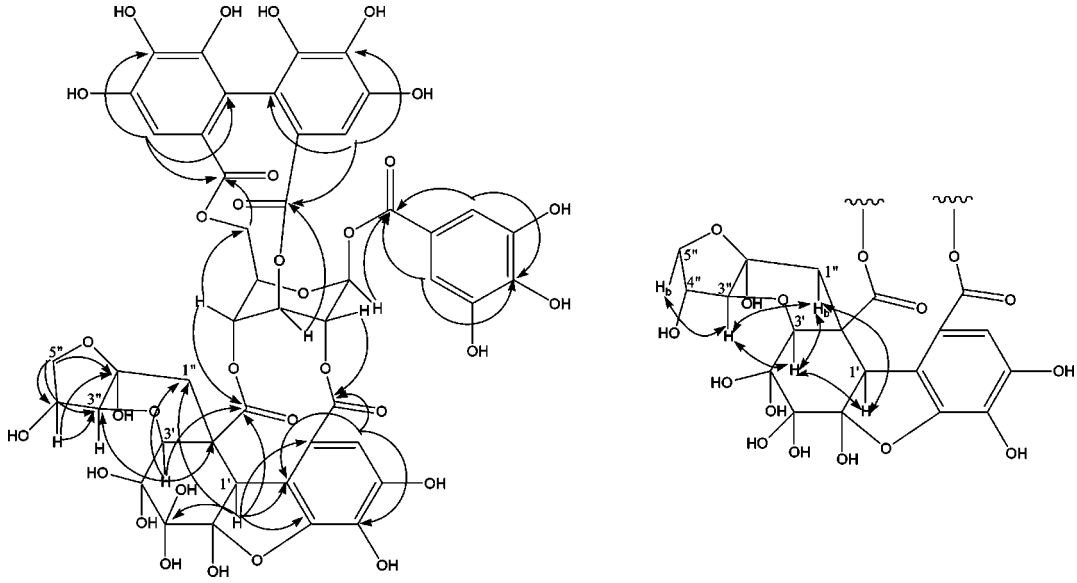

Figure 1. Key HMBC (→) and NOESY (↔) correlations of **1**.

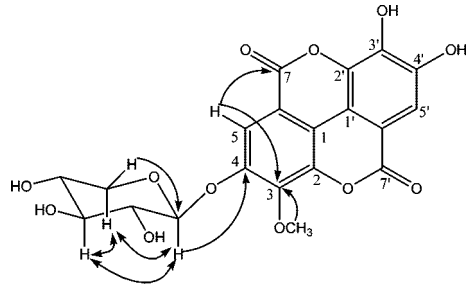

Figure 2. Most relevant HMBC (→) and NOESY (↔) correlations of **2**.

Table 2. Respiratory Burst Inhibition in Human Neutrophils by Compounds **1**−**5** and **7**

compound	inhibition [%] at 1000 μg/mL drug concentration	IC_{50} [μg/mL][a] ± SEM[b]
1	73.23	821.21 ± 73.30
2	29.05	
3	27.10	
4	13.70	
5	42.90	
7	31.20	
indomethacin[c]	58.82	757.99 ± 5.90
aspirin[c]	70.45	279.44 ± 4.42

[a] Drug concentration at which 50% inhibition of superoxide radicals was observed relative to blank. [b] Standard mean error of five replicates. [c] Positive control.

column chromatography using MeOH as eluent to yield **2** (68 mg), **3** (36 mg), **4** (42 mg), and **6** (97 mg). From F_5 and F_8, compounds **5** (30 mg) and **7** (18 mg) were obtained by preparative TLC on silica gel plates using 5% $CHCl_3$−MeOH. The more polar fraction from the fractionation of F_8 furnished **1** (15 mg) by recycling preparative HPLC using a H_2O−MeOH (1:1) mixture with a retention time of 16 min.

Macabarterin (1): brown, amorphous powder; $[\alpha]^{30}_D$ +60.6 (*c* 0.033, MeOH); IR (KBr) ν_{max} 3414, 1727, 1602, 1440, 1343, 1213, and 1030 cm^{-1}; ^1H (400 MHz, MeOH) and ^{13}C NMR (100 MHz, MeOH) data (see Table 1); HRESIMS *m/z* 1103.1621 $[M + H]^+$ (calcd for $C_{42}H_{38}O_{32}$, 1103.1655).

3-*O*-Methyllellagic acid 4-*O*-*β*-D-xylopyranoside (2): white, amorphous powder; $[\alpha]^{30}_D$ +15.4 (*c* 0.130, DMSO); IR (KBr) ν_{max} 3417, 1735, 1613, 1485, 1436, 1352, 1244, 1180, 1108, and 1053 cm^{-1}; ^1H (500 MHz, DMSO) and ^{13}C NMR (125 MHz, DMSO) data, see Table 1; HRESIMS: *m/z* 449.1165 $[M + H]^+$ (calcd for $C_{20}H_{16}O_{12}$, 449.1198). Hydrolysis yielded D-xylose and 3-*O*-methylellagic acid (**4**).

Bioassay Procedure. Isolation of Human Neutrophils. Human neutrophils were isolated by the modified method of Siddiqui et al.[25] Briefly, fresh heparinized blood was collected from healthy volunteers and diluted with an equal volume of modified Hank's solution (MHS), pH 7.4. After leaving for 20 min at room temperature, the upper leukocyte layer was collected, layered over Ficoll paque (Pharmacia Biotech., Uppsala, Sweden), and centrifuged at 1500 rpm, and the pellets were resuspended with MHS. A cell count was performed by using the improved Neubaur chamber. The viability of cells determined by the Trypan Blue method was above 97%.

Respiratory Burst Inhibitory Assay. The respiratory burst inhibitory activities of **1**−**5** and **7** were determined by means of the modified assay of Tan and Berridge.[20] This *in vitro* assay is based on the reduction of highly water-soluble tetrazolium salt (WST-1) in the presence of activated neutrophils. Anti-inflammatory activity was determined in a total volume of 200 μL of MHS (pH 7.4) containing 1.0×10^4 neutrophils/mL, 250 μM WST-1, and various concentrations of test compounds. The control contained buffer, neutrophils, and WST-1. All compounds were equilibrated at 37 °C, the reaction was initiated by adding opsonized zymosan A (15 mg/mL), which was prepared by mixing with human pooled serum, followed by centrifugation at 3000 rpm, and the pellet was resuspended in PBS buffer. Absorbance was measured at 450 nm. Aspirin and indomethacin were used as positive controls. IC_{50} values were calculated by comparison with DMSO as the blank and expressed as the percent inhibition of superoxide anions produced.

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