Madhucosides A and B, Protobassic Acid Glycosides from *Madhuca indica* with Inhibitory Activity on Free Radical Release from Phagocytes

Rahul S. Pawar and K. K. Bhutani*

Department of Natural Products, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, SAS Nagar, Punjab 160062, India

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The structures of madhucosides A (**1**) and B (**2**), isolated from the bark of *Madhuca indica*, were established as 3-*O*-*β*-D-apiofuranosyl(1→2)-*β*-D-glucopyranosyl-28-*O*-{*β*-D-xylopyranosyl(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranosyl(1→ 3)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl}protobassic acid and 3-*O*-*β*-D-apiofuranosyl(1→2)-*β*-D-glucopyranosyl-28-*O*-{*β*-D-xylopyranosyl(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl}protobassic acid, respectively. These two compounds showed significant inhibitory effects on both superoxide release from polymorphonuclear cells in a NBT reduction assay and hypochlorous acid generation from neutrophils assessed in a luminol-enhanced chemiluminescence assay.

Madhuca indica J. F. Gmel (syn. *Madhuca latifolia, Bassia latifolia*) (Sapotaceae) is a tree widely distributed throughout India. The bark is used traditionally in the treatment of rheumatism, ulcers, tonsillitis, and diabetes mellitus. The presence has been reported of various sterols, esters of triterpene acids, and phenolic compounds[1−3] along with two bassic acid glycosides.[4] The related species, namely, *M. longifolia*[5] and *M. butyracea*,[6,7] are reported to contain various protobassic acid glycosides. In the present note, two compounds (**1** and **2**), with a protobassic acid skeleton and containing seven saccharide units at C-3 and C-28, are reported for the first time from this species. Both the compounds showed in vitro inhibitory activity on the release of superoxides from polymorphonuclear cells and hypochlorous acid from neutrophils.

The ESIMS of **1** indicated a molecular weight of 1486 (calcd for $C_{68}H_{110}O_{35}$) with a $[M + Na]^+$ ion at *m*/*z* 1509 and a $[MH + Na - OH]^+$ ion at *m*/*z* 1493. The fragmentation pattern as depicted in Figure S1 (Supporting Information) indicated the loss of four pentoses, a hexose, and two rhamnose moieties. The IR spectrum showed absorbances at 3406 (hydroxyl), 1732 (ester bond), and 1649 and 804 cm^{-1} (trisubstituted double bond). The sugar portion obtained on acid hydrolysis (1 N HCl) of **1** showed the presence of arabinose, rhamnose, glucose, xylose, and an unknown sugar by co-TLC with authentic sugars. The unknown sugar was identified as apiose from NMR studies that showed the presence of two characteristic quaternary carbons at δ_C 80.06 and 80.6 for C-3 and methylenes at δ_C 74.8 and 74.9 for C-4.[7] These sugars and their configurations were further confirmed by HPLC analysis.

The ^1H NMR spectrum (500 Mz) of **1** showed the presence of eight methyls, a multiplet at δ 5.22 for a vinylic proton, and a doublet at δ_H 3.52 ($J = 4$ Hz) for glycosylated H-3. The anomeric region of the ^1H NMR spectrum further showed doublets for seven sugars at δ_H 4.37 ($J = 7$ Hz) for glucose, δ_H 4.53 ($J = 7$ Hz) for xylose, δ_H 4.80 ($J = 1$ Hz) for rhamnose, δ_H 5.02 ($J = 1$ Hz) for a second rhamnose, δ_H 5.15 ($J = 3$ Hz) for apiose, δ_H 5.18 ($J = 3$ Hz) for a second apiose, and δ_H 5.60 ($J = 3$ Hz) for arabinose. The ring protons of the monosaccharide units were assigned starting from the readily identifiable anomeric protons by means of the COSY, HSQC, and HMBC spectra. The ^{13}C NMR assignments, aided by DEPT and HSQC studies, as shown in Table 1, indicated the presence of eight methyls, 16 methylenes, 34 methines, and 10 quaternary carbon atoms. The double-bond carbons appeared at δ_C 124.0 and 145.0,

Table 1. ^{13}C NMR Data of **1** and **2** Recorded at 125 Mz and of **4** at 75 Mz in CD_3OD

carbon	**1**	**2**	**4**		**1**		**2**
C-1	47.1	47.1	47.3	ara 1	93.5	ara 1	94.0
C-2	70.1	70.0	71.2	2	76.2	2	75.6
C-3	83.8	83.9	83.9	3	71.3	3	71.2
C-4	43.1	43.1	43.2	4	66.1	4	65.9
C-5	49.6	49.5	49.6	5	62.2	5	62.2
C-6	69.1	69.4	69.7	rha 1	101.1	rha 1	101.3
C-7	44.4	44.3	44.3	2	81.8	2	72.2
C-8	40.7	40.7	40.6	3	72.2	3	75.1
C-9	48.3	48.3	74.8	4	73.9	4	74.1
C-10	37.5	37.5	37.5	5	69.6	5	69.6
C-11	24.7	24.7	24.7	6	17.6	6	17.6
C-12	124.0	123.9	123.6	xyl 1	105.0	glc 1	103.2
C-13	145.0	144.9	145.5	2	79.0	2	83.6
C-14	43.1	43.1	42.8	3	84.0	3	74.9
C-15	28.8	29.0	28.8	4	70.0	4	80.3
C-16	23.7	23.7	24.0	5	66.9	5	77.9
C-17	48.3	48.1	48.3	api 1	112.6	6	62.3
C-18	43.1	42.6	41.1	2	77.9	xyl 1	106.5
C-19	48.1	48.3	48.1	3	80.6	2	77.6
C-20	31.6	31.6	31.6	4	74.8	3	77.7
C-21	34.9	34.9	35.0	5	64.9	4	74.0
C-22	33.4	33.2	33.5	rha 1	102.5	5	67.0
C-23	65.7	65.7	65.6	2	71.9	rha 1	102.2
C-24	14.8	14.9	14.7	3	73.9	2	72.4
C-25	17.8	17.9	17.9	4	74.9	3	74.0
C-26	17.9	17.9	17.5	5	69.8	4	74.9
C-27	26.5	26.4	26.5	6	18.0	5	69.0
C-28	177.8	177.9	182.5			6	18.0
C-29	33.5	33.5	33.6				
C-30	24.1	24.0	24.0				
glc 1	105.3	105.3	105.4				
2	85.8	85.9	85.9				
3	78.3	78.2	78.2				
4	71.1	71.1	71.2				
5	78.2	78.1	77.9				
6	62.6	62.8	62.2				
api 1	111.3	111.3	111.4				
2	77.4	77.1	77.4				
3	80.6	80.5	80.5				
4	74.9	74.8	75.0				
5	65.1	65.1	65.1				

* To whom correspondence should be addressed. Tel: 91-172-214682, -214695. Fax: 91-172-214692. E-mail: kkbhutani@usa.net.

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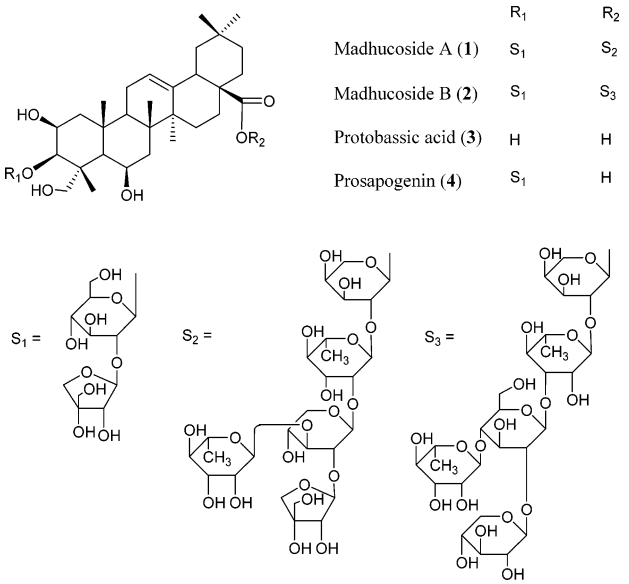
and a quaternary carbon at δ_C 177.8 was assignable for an ester. Further, hydroxylated carbons for C-2, C-6, and C-23 appearing at δ_C 70.1, 69.1, and 65.7, respectively, and the glycosylated C-3 carbon at δ_C 83.8 suggested the presence of a triterpenoidal protobassic acid aglycon in the molecule. Figure S2 (Supporting Information) indicates the long-range correlations observed in the HMBC experiment that permitted the assignment of protobassic acid (**3**).[8]

A prosapogenin (**4**) obtained on basic hydrolysis (5% KOH) of **1** exhibited a molecular weight of 798. In the ^{1}H NMR spectrum (300 Mz), signals for the six methyls of the aglycon skeleton were observed, indicating that two rhamnose moieties were attached to the aglycon through an ester bond. The ^{1}H NMR spectrum further exhibited two anomeric protons at δ_H 4.30 (J = 7 Hz) for glucose and δ_H 5.18 (J = 3 Hz) for apiose. The ^{13}C NMR spectrum of **4** showed six methyls, 12 methylenes, 14 methines, and nine quaternary carbons. The signals at δ_C 182.5, 105.4, and 111.4 were assignable to the quaternary carbon of the acid group and the anomeric carbons of the glucose and apiose units, respectively. In the HMBC spectrum of **1**, the long-range coupling between the anomeric proton of glucose (δ_H 4.30) and δ_C 83.9 at the C-3 position of the aglycon confirmed that a glucose unit was attached to the protobassic acid unit at C-3. The ^{13}C NMR values showed a downfield shifted C-2 carbon of glucose at δ_C 85.9, which further showed a long-range correlation with the anomeric proton of apiose at δ_H 5.18. Hence, the prosapogenin **4** was characterized as 3-*O*-β-D-apiofuranosyl(1→2)-β-D-glucopyranosylprotobassic acid, a new compound.



On the alkaline hydrolysis of **1**, it was clear that the sugar chain at the C-28 position containing five saccharides was cleaved. The linkages between the saccharides were established using HMBC data (Figure S2). The HMBC correlation between the anomeric proton of arabinose δ_H 5.60 and δ_C 177.8 for the C-28 carbonyl of the protobassic acid skeleton further corroborated the attachment of arabinose to C-28 of the protobassic acid unit. The arabinose moiety showed one downfield carbon at δ_C 76.2, indicating a single saccharide substitution. An anomeric proton at δ_H 4.80 for a rhamnose was correlated with the C-2 position of arabinose at δ_C 76.2. The rhamnose exhibited one downfield carbon at δ_C 81.8 attached to a proton at δ_H 3.77 that was correlated with a signal at δ_C 105.0, the anomeric proton of a xylose unit. This xylose unit had two downfield shifted carbons at δ_C 79.0 and 83.9 that were correlated with the anomeric proton. The proton at δ_H 3.95 attached to the carbon at δ_C 79.0 was correlated with the anomeric proton of apiose at δ_C 112.6. Further, the proton appearing at δ_H 3.34 that was attached to the second downfield carbon at δ_C 83.9 showed a correlation with δ_C 102.5, an anomeric carbon for another rhamnose. The sequence of sugar units fragmented in the ESIMS further corroborated the linkage of sugars in **1**. Accordingly, the structure of madhucoside A (**1**) was determined as 3-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranosyl-28-*O*-{β-D-apiofuranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→3)]-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl}protobassic acid, a new compound.

The ESIMS of **2** showed a molecular weight of 1516 (calcd for $C_{69}H_{112}O_{36}$) with a $[M + Na]^+$ ion at m/z 1539 and a $[MH + Na - OH]^+$ ion at m/z 1523. The ESIMS fragmentations as depicted in Figure S1 (Supporting Information) showed fragments for the losses of two hexoses, two rhamnoses, and three pentoses, suggesting that one of the pentose units in **1** was replaced by a hexose in **2**. The hexose was lost only after the successive loss of a rhamnose and a pentose unit, suggesting that the hexose is not a terminal sugar moiety. Thus, based on the fragmentation pattern, it was clear that sugars in both these compounds differed in composition as well as in their linkage. The sugar portion obtained on acid hydrolysis of **2** showed the presence of arabinose, rhamnose, glucose, xylose, and apiose. The IR spectrum of **2** was similar to that of **1**. The ^{1}H NMR of **2** showed the presence of eight methyls, and the anomeric region showed doublets for seven sugars at δ_H 4.37 (J = 7 Hz) for glucose, δ_H 4.10 (J = 7 Hz) for a second glucose, δ_H 4.40 (J = 7 Hz) for xylose, δ_H 4.90 (J = 1 Hz) for rhamnose, δ_H 5.06 (J = 1 Hz) for a second rhamnose, δ_H 5.19 (J = 3 Hz) for apiose, and δ_H 5.50 (J = 3 Hz) for arabinose. The ^{13}C NMR spectrum aided by DEPT and HSQC data indicated the presence of eight methyls, 15 methylenes, 37 methines, and nine quaternary carbon atoms. Further, carbon signals for a double bond, an ester carbonyl, and four hydroxylated carbons suggested the presence of a protobassic acid aglycon similar to that of **1**. The prosapogenin (**4**) obtained on alkaline hydrolysis of **2** was found to be identical to that of **1** in the co-TLC and spectral studies.

The ^{13}C NMR spectrum of **2** showed an anomeric proton at δ_C 94.0 for an arabinose unit attached to the C-28 position of protobassic acid. The HMBC correlations depicted in Figure S2 (Supporting Information) confirmed this fact. Further, this arabinose unit showed a single downfield shifted carbon at δ_C 75.6 that was correlated with the anomeric proton of rhamnose at δ_H 4.90. The same anomeric proton was correlated with the signals of the glycosylated C-3 position at δ_C 75.1. The anomeric proton for a second glucose at δ_H 4.10 was further correlated with the C-2 position of rhamnose, indicating that glucose was attached to rhamnose at the C-2 position. This glucose showed two downfield shifted carbon signals at δ_C 80.3 and 83.6. An anomeric proton at δ_H 4.40 for xylose showed a correlation with the signal at δ_C 83.6, and another anomeric proton at δ_H 5.06 for rhamnose showed a correlation with the signal at δ_C 80.3. This indicated that xylose and rhamnose units were attached to the glucose unit at the C-2 and C-4 positions, respectively. On the basis of the above analysis, the new compound madhucoside B (**2**) was assigned a structure of 3-*O*-β-D-apiofuranosyl(1→2)-β-D-glucopyranosyl-28-*O*-{β-D-xylopyranosyl(1→2)-[α-L-rham-

nopyranosyl(1→4)]-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl}protobassic acid.

In an in vitro luminol-enhanced chemiluminescence assay, compounds **1** and **2** inhibited the production of hypochlorous acid by human neutrophils with an IC_{50} of 21.9 and 144 μg/mL, respectively, compared with the IC_{50} of 19.84 μg/mL of bacoside A_3, used as reference standard.[9] Further, in the NBT reduction assay used for the quantification of superoxide production from polymorphonuclear cells, **1** was found to have an inhibitory effect with an IC_{50} of 138.3 μg/mL, as compared to the IC_{50} values of 111.0 and 14.1 μg/mL for two reference standards, quercetin and ascorbic acid, also used in the experiment.[10] However, only 16% inhibition was achieved in the case of **2** at the highest tested concentration of 200 μg/mL.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Autopol IV polarimeter. IR spectra were recorded on an Impact 410 Nicolet infrared spectrometer. The NMR spectra for saponins **1** and **2** were recorded on a JEOL 500 Mz NMR spectrophotometer, while those of prosapogenin **4** were obtained on a Bruker DPX 300 instrument. ESIMS studies were done on Finnigan MATT LCQ and HRESIMS on a Bruker Daltonics mass spectrometer.

Plant Material. The bark of *M. indica* was collected in Chandigarh, India, in December 2001 and was identified by Mr. A. S. Sandhu, NIPER. A voucher specimen (NIP-73) has been deposited in the NIPER herbarium.

Extraction and Isolation. The milled bark (6.0 kg) was defatted with *n*-hexane (3 × 10 L) and then exhaustively extracted with methanol (9 × 7 L). The marc was further extracted with deionized water (3 × 8 L), and the extract was freeze-dried. The aqueous extract (550 g) was redissolved in water and exhaustively extracted with *n*-butanol (10 × 500 mL). The concentrated *n*-butanol fraction was detannified with lead acetate and further subjected to column chromatography on silica gel using gradients of chloroform−methanol (80:20; 70:30) and chloroform−methanol−water (75:30:5) for elution. The fractions obtained from the elution with chloroform−methanol−water were pooled and rechromatographed on silica gel using ethyl acetate−methanol−water (80:10:10) elutions to obtain two crude saponins. These were further purified by passing through Sephadex LH-20 (MeOH) and column chromatography on reversed-phase C_{18} by elution with mixtures of methanol−water (1:1 to 7:3) to provide madhucoside A (**1**, 30 mg) and madhucoside B (**2**, 45 mg).

Madhucoside A (1): colorless amorphous powder (MeOH); $[\alpha]^{27}_D$ −38.8° (*c* 1, MeOH); IR (KBr) ν_{max} 3406, 2929, 1732, 1649, 1456, 1386, 1260, 1071, 1045, 804 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 0.70 (3H, s, H-26), 0.81 (3H, s, H-29), 0.85 (6H, s, H-24, H-30), 1.04 (2H, m, H-15), 1.07 (3H, s, H-27), 1.14 (3H, d, *J* = 6.1 Hz, rha-Me), 1.15 (3H, d, rha-Me), 1.19 (3H, s, H-25), 1.21 (1H, s, H-5), 1.59 (2H, m, H-16), 1.98 (2H, br s, H-1), 3.52 (1H, d, *J* = 4 Hz, H-3), 4.37 (1H, d, *J* = 7.3 Hz, glc H-1), 4.53 (1H, d, *J* = 7.6 Hz, xyl H-1), 4.80 (1H, d, *J* = 1 Hz, rha H-1), 5.02 (1H, d, *J* = 1 Hz, rha H-1), 5.15 (1H, d, *J* = 3 Hz, api H-1), 5.18 (1H, d, *J* = 3 Hz, api H-1), 5.22 (1H, m, H-12), 5.60 (1H, d, *J* = 3.0 Hz, ara H-1); ^{13}C NMR (CD_3OD), see Table 1; ESIMS (positive-ion mode) *m/z* 1509 $[M + Na]^+$, 1493 $[MH + Na - OH]^+$; HRESIMS *m/z* 1509.6616 $[M + Na]^+$ (calcd for $C_{68}H_{110}O_{35}Na$, 1509.6719).

Madhucoside B (2): colorless amorphous powder (MeOH); $[\alpha]^{27}_D$ −25.4° (*c* 1, MeOH); IR (KBr) ν_{max} 3421, 2931, 1734, 1652, 1456, 1386, 1260, 1071, 1048 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 0.70 (3H, s, H-26), 0.81 (3H, s, H-29), 0.84 (3H, s, H-24), 0.85 (3H, s, H-30), 1.04 (2H, m, H-15), 1.07 (3H, s, H-27), 1.18 (3H, d, *J* = 6 Hz, rha-Me), 1.20 (6H, br s, H-25, rha-Me), 1.22 (1H, s, H-5), 1.59 (2H, m, H-16), 1.96 (br s, H-1), 3.51 (1H, d, *J* = 4 Hz, H-3), 4.10 (1H, d, *J* = 7 Hz, glc H-1), 4.37 (1H, d, *J* = 7.3 Hz, glc H-1), 4.40 (1H, d, *J* = 7 Hz, xyl H-1), 4.90 (1H, d, *J* = 1 Hz, rha H-1), 5.06 (1H, d, *J* = 1 Hz, rha H-1), 5.18 (1H, d, *J* = 3 Hz, api H-1), 5.21 (1H, m, H-12), 5.50 (1H, d, *J* = 2.5 Hz, ara H-1); ^{13}C NMR (CD_3OD), see Table 1; ESIMS (positive-ion mode) *m/z* 1539 $[M + Na]^+$, 1523 $[MH + Na - OH]^+$; HRESIMS *m/z* 1539.6839 $[M + Na]^+$ (calcd for $C_{69}H_{112}O_{36}Na$, 1539.6825).

3-*O*-β-D-Apiofuranosyl(1→2)-β-D-glucopyranosylprotobassic acid (4): colorless amorphous powder (MeOH); $[\alpha]^{27}_D$ −69.0° (*c* 0.16, MeOH); IR (KBr) ν_{max} 3410, 2931, 1680, 1652, 1456, 1386, 1260, 1071, 1048 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 0.70 (3H, s, H-26), 0.83 (3H, s, H-29), 0.87 (3H, s, H-24), 0.90 (3H, s, H-30), 0.93 (2H, m, H-15), 1.16 (3H, s, H-27), 1.20 (3H, br s, H-25), 1.22 (1H, s, H-5), 1.59 (2H, m, H-16), 1.96 (br s, H-1), 3.51 (1H, d, *J* = 4 Hz, H-3), 4.30 (1H, d, *J* = 7 Hz, glc H-1), 5.18 (1H, d, *J* = 3 Hz, api H-1), 5.21 (1H, m, H-12); ^{13}C NMR (CD_3OD), see Table 1; ESIMS (positive-ion mode) *m/z* 821 $[M + Na]^+$.

Acid Hydrolysis of 1 and 2. An aqueous solution of each saponin (5 mg) in 1 N HCl was refluxed for 1 h. The excess HCl was removed under vacuum, and the sugars and aglycon were separated by liquid−liquid partitioning between chloroform and water. The sugars thus obtained were subjected to HPLC analysis: Waters μBondapak NH_2 3.9 mm i.d. × 300 mm; detection, ultraviolet 210 nm (Waters PDA detector); mobile phase, CH_3CN-H_2O (75:25); flow 1.0 mL/min; column temperature, room temperature. Individual sugars were obtained by pooling the respective elutes, and their optical rotations were measured on a polarimeter. Identification of D-apiose, l-rhamnose, D-xylose, L-arabinose, and D-glucose was carried out by comparison of its retention time and optical rotation with that of an authentic sample, t_R: 8.1 min (D-apiose, positive optical rotation), 8.6 min (L-rhamnose, positive optical rotation), 9.6 min (D-xylose, positive optical rotation), 10.8 min (L-arabinose, positive optical rotation), 13.0 min (D-glucose, positive optical rotation), respectively.

Alkaline Hydrolysis. Each saponin (5 mg) was refluxed with 5% KOH for 3 h. The reaction mixture was neutralized with dilute HCl and then extracted with *n*-butanol. The prosapogenin (**4**) in the organic layer was purified by column chromatography in silica gel and eluted with chloroform−methanol. The aqueous layer was freed of salts and further hydrolyzed and analyzed as described for acid hydrolysis.

Luminol-Enhanced Chemiluminescence Assay. Human neutrophils were isolated from fresh, herparinized human blood by using Histoprep and suspended in HBSS medium containing gelatin. To the cell suspension (1 × 10^6 cells/mL) was added luminol solution so as to attain the concentration of 0.5 mM of luminol. The cell suspension containing luminol (100 μL) was added to the microtiter plates containing different concentrations of test compounds in triplicate. After 5 min, 10^{-7} M phorbol 12-myristate, 13-acetate (50 μL) was added to trigger the respiratory burst. The luminescence produced was measured in kinetic mode for 90 min, during which each well was read for 740 ms. A curve of light intensity (RLU) was plotted against time, and the area under the curve (AUC) was calculated as total luminescence. The percent inhibition of luminescence was calculated as % inhibition of luminescence = (control − sample)/control × 100.

NBT Reduction Assay. The method of Richardson et al.[11] was followed in which the cell suspension (50 μL) was incubated with different concentrations of the extract/fraction/compound in triplicate in a microcentrifuge tube for 5 min. For the control the cells were incubated with the medium alone. Nitroblue tetrazolium (NBT) (200 μL) and opsonized zymosan (100 μL) were added and incubated at 37 °C. After 30 min of incubation the reaction was quenched by the addition of 1 mL of 1 N HCl. To the cell pellet obtained by centrifugation was added pyridine and heated on a water bath for 30 min at 70 °C. The pyridine extract of the cell mass was transferred to microtiter plates, and absorbance was measured at 515 nm on an ELISA plate reader. The percentage inhibition of NBT reduction was calculated as % inhibition of NBT reduction = (control − sample)/control × 100. The IC_{50} was calculated by Sigmaplot software.

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Supporting Information Available: Figures showing the ESIMS fragmentation pattern and HMBC data for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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