Prenylated Flavonoids from the Heartwood of *Artocarpus communis* with Inhibitory Activity on Lipopolysaccharide-Induced Nitric Oxide Production

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Received January 23, 2006

A new prenylated chalcone, 3″,3″-dimethylpyrano[3′,4′]2,4,2′-trihydroxychalcone (**1**), was isolated from the heartwood of *Artocarpus communis*. Two flavonoid derivatives, (−)-cycloartocarpin (**9**) and (−)-cudraflavone A (**10**), were isolated as new isomers. In addition, eight known flavonoids, isobacachalcone (**2**), morachalcone A (**3**), gemichalcones B (**4**) and C (**5**), artocarpin (**6**), cudraflavone C (**7**), licoflavone C (**8**), and (2*S*)-euchrenone a_7 (**11**), were isolated and identified from this plant for the first time. Compounds **1**−**4**, **6**, and **11** exhibited potent inhibitory activity on nitric oxide production in RAW264.7 LPS-activated mouse macrophage cells with IC_{50} values of 18.8, 6.4, 16.4, 9.3, 18.7, and 12.3, μM, respectively. The structure of compound **1** was elucidated by spectroscopic data analysis, including 1D and 2D NMR experiments.

Artocarpus species (Moraceae) are timber and fruit trees distributed throughout tropical regions of Asia. In particular, *Artocarpus communis* Forst. (breadfruit) is well-known in the Pacific islands for its large edible fruits.[1] Previous phytochemical work on the various parts of *A. communis* has resulted in the isolation of various types of prenylated flavonoids and xanthones.[2−10] Some of the flavonoids isolated from this plant have been demonstrated as 5-lipoxygenase inhibitors,[11] free radical scavengers, and antinephritis agents.[12]

During a screening procedure on higher plants to find novel candidates as inducible nitric oxide synthase (iNOS) inhibitory agents, the chloroform extract of the heartwood of *A. communis* was shown to exhibit considerable inhibitory activity (IC_{50} = 10.0 μg/mL). Therefore, this chloroform extract (3.5 g) was subjected to detailed laboratory investigation. In the present study, eleven compounds (**1**−**11**) including a new chalcone (**1**) were isolated and were evaluated for their inhibitory activity on nitric oxide (NO) production in RAW 264.7 mouse macrophage cells.

Compound **1** gave a molecular ion peak at *m*/*z* 339.1232 [M + H]$^+$ in the HRFABMS, corresponding to the elemental formula $C_{20}H_{19}O_5$. The IR spectrum showed absorption bands at 3346 cm^{-1} for one or more hydroxyl groups and 1707 cm^{-1} for a conjugated carbonyl functionality. The UV spectrum of **1** exhibited absorption maxima at 273 and 386 nm, indicating the presence of aromatic rings.[13] The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of the known chalcones 4-hydroxylonchocarpin and muncericin.[14] The differences between the NMR spectroscopic data of compound **1** and 4-hydroxylonchocarpin were in the signals of the B-ring. On the other hand, the A-ring pattern was different between compound **1** and muncericin. In the ^{1}H NMR spectrum of **1**, the signals at δ_H 7.51 (1H, d, J = 8.6 Hz, H-6), 6.36 (1H, dd, J = 8.6, 2.2 Hz, H-5), and 6.34 (1H, d, J = 2.2 Hz, H-3) showed the presence of a 2,4-disubstituted B-ring system in the chalcone moiety, which was supported by the HMBC NMR correlations of H-α/C-1, H-β/C-2,C-6, H-3/C-2,C-4, H-5/C-1, and H-6/C-5. Further detailed analysis of ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC NMR data (Figure 1) allowed unambiguous assignments for all of the ^{1}H and ^{13}C NMR signals of **1**.

Compounds **2**−**8** were identified as isobacachalcone (**2**),[15] morachalcone A (**3**),[16] gemichalcone B (**4**),[17] gemichalcone C (**5**),[18] artocarpin (**6**),[19,20] cudraflavone C (**7**),[21] licoflavone C (**8**),[22] respectively, by physical and spectroscopic methods as well as by comparison of their data with those of published values.

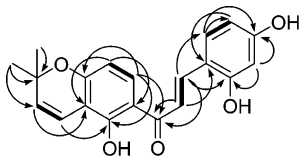

Figure 1. Important ^{1}H−^{1}H COSY (—) and ^{1}H−^{13}C HMBC (→) correlations of compound **1**.

Compounds **9** and **10** exhibited NMR spectra identical to those of (+)-cycloartocarpin[23] and (+)-cudraflavone A,[24] respectively. (+)-Cudraflavone A was previously found from *A. communis*. However, these compounds showed different optical rotations from the literature values. The $[\alpha]_D$ values of **9** and **10** were −159.8 (*c* 0.34, MeOH; literature values: +1.1, *c* 0.02, MeOH;[23] +1.19, *c* 0.225, $CHCl_3$[25]) and −40.5 (*c* 0.35, MeOH; literature values: +27.3, *c* 0.225, $CHCl_3$;[24] +30.0, *c* 0.225, $CHCl_3$[26]), respectively. Therefore, compounds **9** and **10** were determined as the new isomers (−)-cycloartocarpin and (−)-cudraflavone A, respectively. Compound **11** was identified as the known flavanone (2*S*)-euchrenone a_7 (**11**) by comparison of its NMR data with published values.[27] The configuration at C-2 in the molecule of **11** was determined as 2*S* due to its CD spectrum [positive Cotton effects at 312 and 278 nm (n → π* transition) and a negative Cotton effect at 290 nm (π → π* transition)].[28] To the best of our knowledge, the known compounds **2**−**8** and **11** have been isolated from *A. communis* for the first time by the present study. Moreover, this is the first report on compounds **2**, **5**, **8**, and **11** from the genus *Artocarpus*.

The inducible NOS (iNOS) inhibitory assay was performed by measuring NO production in RAW 264.7 LPS-induced mouse macrophage cells. The inorganic free radical nitric oxide (NO) has been implicated in physiological and pathological processes such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation.[29] NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). Among the NOS family, iNOS, in particular, is involved in overproduction of NO associated with oxidative stress and with the pathophysiological responses including circulatory shock, inflammation, and carcinogenesis.[30] Also, it can be expressed in response to pro-inflammatory agents such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS) in

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10.1021/np0600346 CCC: $33.50
Published on Web 03/18/2006

Chart 1

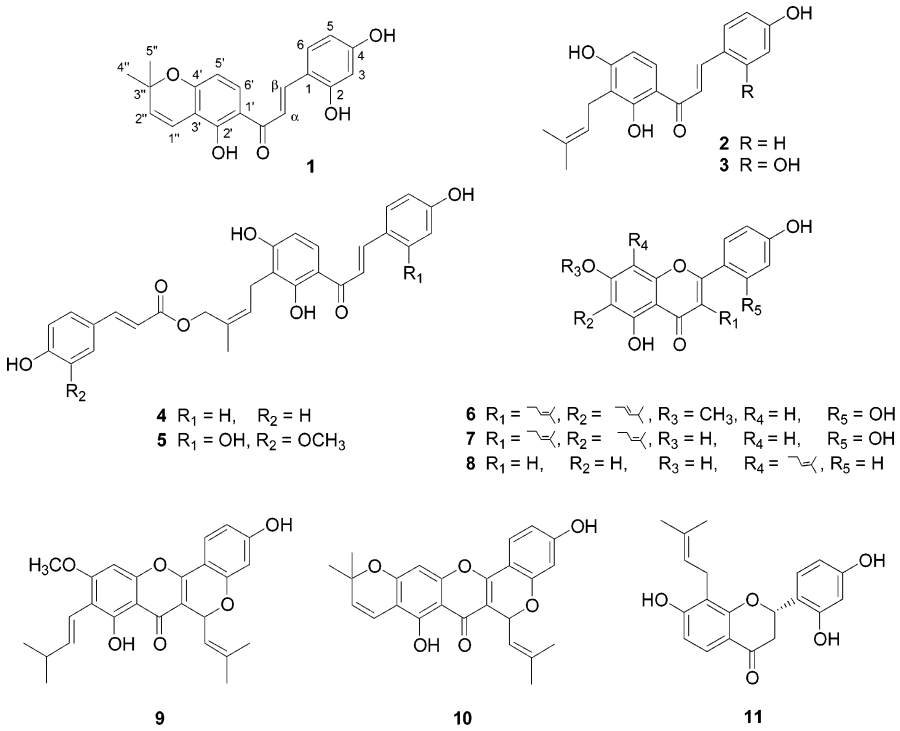

Table 1. Inhibitory Effects of Compounds **1**−**11** on NO Production on RAW264.7 LPS-Activated Mouse Macrophage Cells

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	L-NMMA[a]
IC_{50} (μM)	18.8	6.4	16.4	9.3	23.9	18.7	52.3	20.4	>60	42.1	12.3	19.2
cytotoxicity IC_{50} (μM)	59.2	16.4	58.0	35.1	37.6	45.3	59.2	41.6	46.1	42.1	47.9	>50
SI value[b]	3.1	2.6	3.5	3.8	1.6	2.4	1.1	2.0	<0.8	1	3.9	>2.6

[a] N^{ω}-Monomethyl-L-arginine (L-NMMA) was used as a positive control. [b] Selective index value: CC (cytotoxic concentration)$_{50}$/IC_{50}.

various cell types including macrophages, endothelial cells, and smooth muscle cells.[31,32]

Compounds **1**−**4**, **6**, and **11** exhibited potent inhibitory activity on nitric oxide production in LPS-activated mouse macrophage RAW264.7 cells with IC_{50} values of 18.8, 6.4, 16.4, 9.3, 18.7, and 12.3 μM, respectively, as shown in Table 1. In particular, compounds **1**, **3**, **4**, and **11** showed favorable selectivity indices (SI = 3.1, 3.5, 3.8, and 3.9, respectively), which are indicative of their iNOS inhibitory activity without cytotoxicity. Compounds **5** and **8** showed inhibitory activity with IC_{50} values of 23.9 and 20.4 μM, respectively, but their inhibitory activity on NO production seems to be related to their concomitant cytotoxic effects (SI = 1.6 and 2.0, respectively). Compounds **7**, **9**, and **10** were weakly active or inactive in the present study.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1010 polarimeter at 25 °C. UV and IR spectra were recorded on a Hitachi U-3000 spectrophotometer and a Bio-Rad FTS 135 FT-IR spectrometer, respectively. 1D and 2D NMR experiments were performed on a Varian Unity INOVA 400 MHz FT-NMR instrument with tetramethylsilane (TMS) as internal standard. EIMS and FABMS were obtained on a JEOL JMS 700 Mstation HRMS spectrometer operating at 70 eV. Silica gel (230−400 mesh, Merck, Germany), RP-18 (ODS-A, 12 nm, S-150 μm, YMC, Japan), and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (silica gel, 0.25 mm layer thickness, Merck, Germany) and RP-18 F_{254s} (Merck, Germany) plates, with visualization under UV light (254 and 365 nm) and 10% (v/v) sulfuric acid spray followed by heating (120 °C, 5 min).

Plant Material. The heartwood of *Artocarpus communis* Forst. (Moraceae) was collected in Surabaya, Indonesia, in June 2001 and was identified by one of the authors (T.W.). A voucher specimen (No. 05/DT/VIII/2001) has been deposited at the University of Surabaya.

Extraction and Isolation. The dried heartwood of *A. communis* (500 g) was extracted with MeOH (3 × 2 L) overnight at room temperature. The solvent was evaporated in vacuo to afford a MeOH extract (11.3 g), which was then suspended in water (200 mL) and partitioned with *n*-hexane (4 × 200 mL), $CHCl_3$ (4 × 200 mL), and *n*-BuOH (2 × 200 mL), sequentially. The *n*-hexane and $CHCl_3$ extracts (3.5 g) were combined due to their overlapping TLC pattern and were separated by flash silica gel column chromatography (ϕ 4.5 cm; 230−400 mesh, 40 g) using gradient mixtures of MeOH in $CHCl_3$ (0 → 20%) as mobile phases, affording 17 fractions (F1−F17). Fraction 6 (260 mg), eluted with 1% MeOH in $CHCl_3$ from the first separation, was subjected again to flash silica gel column chromatography (ϕ 2 cm; 230−400 mesh, 20 g), with *n*-hexane−EtOAc (19:1 → 2:1) as solvent system, providing **9** (12.4 mg, 0.00248%) and **10** (4.2 mg, 0.00084%). Fraction 8 (100 mg), eluted with 1% MeOH in $CHCl_3$ from the first separation, was chromatographed further over Sephadex LH-20 (100% MeOH) and then subjected to preparative TLC (*n*-hexane−EtOAc−formic acid = 7:3:0.05), affording **2** (7.4 mg, 0.00148%). Fractions 9 and 10 (400 mg), eluted with 1−2% MeOH in $CHCl_3$ from the first separation, were combined due to their overlapping TLC pattern. These combined fractions were subjected to reversed-phase column chromatography (ϕ 2.5 cm; ODS-A, 12 nm, S-150 μm, 40 g) using a gradient solvent system of MeOH−H_2O (4:1), giving **6** (29.2 mg, 0.00584%). Passage over Sephadex LH-20 of fraction 11 (110 mg), eluted with 3−5% MeOH in $CHCl_3$ from the first separation, using 100% MeOH, afforded **1** (0.7 mg, 0.00014%) and **4** (2.9 mg, 0.00058%). Fraction 12 (200 mg), eluted with 5% MeOH in $CHCl_3$ from the first separation, was subjected to Sephadex LH-20 column chromatography using 100% MeOH as solvent, yielding **5** (3.4 mg, 0.00068%). The fourth fraction from this column was further fractionated by reversed-phase column chromatography to afford **7** (2.6 mg, 0.00052%), **8** (3.8 mg, 0.00076%), and **11** (0.9 mg, 0.00018%), using MeOH−H_2O (3:1). The seventh

fraction from this column was subjected to passage over Sephadex LH-20, using 100% MeOH as eluent, affording **3** (26.0 mg, 0.0052%).

3″,3″-Dimethylpyrano[3′,4′]2,4,2′-trihydroxychalcone (1): yellow amorphous powder; UV (MeOH) λ_{max} (log ϵ) 273 (3.85), 386 (3.84) nm; IR (film) ν_{max} 3346, 2927, 2852, 1707, 1604, 1454, 1368, 1244, 1115 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 8.12 (1H, d, J = 15.2 Hz, H-β), 7.84 (1H, d, J = 8.8 Hz, H-6′), 7.70 (1H, d, J = 15.2 Hz, H-α), 7.51 (1H, d, J = 8.6 Hz, H-6), 6.71 (1H, d, J = 10.0 Hz, H-1″), 6.36 (1H, dd, J = 8.6, 2.2 Hz, H-5), 6.35 (1H, d, J = 8.8 Hz, H-5′), 6.34 (1H, d, J = 2.2 Hz, H-3), 5.66 (1H, d, J = 10.0 Hz, H-2″), 1.44 (6H, s, H-4″ and H-5″); ^{13}C NMR (CD_3OD, 100 MHz) δ 194.5 (s, C=O), 163.2 (s, C-4), 161.1 (s, C-2′), 160.7 (s, C-2 and C-4′), 142.6 (d, C-β), 132.6 (d, C-6), 132.1 (d, C-6′), 129.4 (d, C-2″), 117.3 (d, C-α), 116.8 (d, C-1″), 116.3 (s, C-1′), 115.6 (s, C-1), 110.4 (s, C-3′), 109.2 (d, C-5), 109.1 (d, C-5′), 103.7 (d, C-3), 78.7 (s, C-3″), 28.6 (q, C-4″ and C-5″); FABMS (positive mode) m/z 339 $[M + H]^+$ (11), 338 (23), 353 (30), 338 (25), 329 (11), 313 (11), 267 (21), 231 (20), 203 (11); HRFABMS (positive mode) m/z 339.1232 $[M + H]^+$ (calcd for $C_{20}H_{19}O_5$, 339.3679).

(−)-Cycloartocarpin (9): $[\alpha]_D^{25}$ −159.8 (*c* 0.34, MeOH).

(−)-Cudraflavone A (10): $[\alpha]_D^{25}$ −40.5 (*c* 0.35, MeOH).

(2*S*)-Euchrenone a_7 (11): $[\alpha]_D^{25}$ −25.0 (*c* 0.12, MeOH); CD (*c* 0.65 mM, MeOH) $\Delta\epsilon_{240}$ −9.5, $\Delta\epsilon_{278}$ +35.9, $\Delta\epsilon_{290}$ −22. 6, $\Delta\epsilon_{312}$ +4.6 nm.

Measurement of NO Production on LPS-Stimulated Macrophage Cells. Measurement of NO formation by iNOS was performed in cultured RAW 264.7 macrophage cells. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin−streptomycin and 10% fetal bovine serum (FBS) at 37 °C, in 5% CO_2 humidified air. To evaluate the inhibitory activity of test materials on LPS-induced NO production, the cells in 10% FBS−DMEM without phenol red were plated in 24-well plates (5×10^5 cells/mL) and then incubated for 24 h. After incubation, the cells were washed with PBS, replaced with new media, and then incubated in the medium with 1 μg/mL of LPS in the presence or absence of test samples. After an additional 20 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction. Briefly, 180 μL of Griess reagent [0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% H_3PO_4] was added to 100 μL of each supernatant from LPS or sample-treated cells in 96-well plates. The absorbance was measured at 540 nm using a microplate reader, and nitrite concentration was determined by comparison with a sodium nitrite standard curve. The percentage inhibition was expressed as [1 − (NO level of test samples/NO level of vehicle-treated control)] × 100. The IC_{50} value, the sample concentration resulting in 50% inhibition of NO production, was determined using nonlinear regression analysis (% inhibition versus concentration). N^{ω}-Monomethyl-L-arginine (L-NMMA) was used as a positive control.

MTT Assay. Compounds **1**−**11** were tested for cytotoxicity using established protocols.[33]

Acknowledgment. This study was supported by a Korea Research Foundation Grant (KRF-2005-005-J01501). We are grateful to the late Prof. Gwang-Ho Jeohn for the plant collection.

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NP0600346