Saponins from *Cussonia bancoensis* and Their Inhibitory Effects on Nitric Oxide Production

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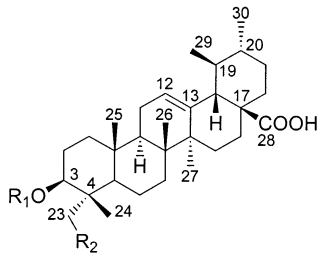
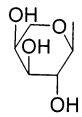
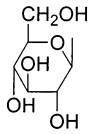
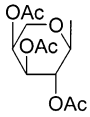
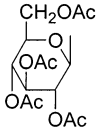
Two new triterpenoid saponins (**1** and **2**) were isolated from the stem bark of *Cussonia bancoensis* together with the known stigmasterol, ursolic acid, 23-hydroxyursolic acid (**3**), and 3*β*-hydroxylup-20(29)-en-28-oic acid. On the basis of their spectroscopic data and on chemical transformations, the structures of the new saponins have been established as 3-*O*-(α-L-arabinopyranosyl)-23-hydroxyursolic acid (**1**) and 3-*O*-(*β*-D-glucopyranosyl)-23-hydroxyursolic acid (**2**). In a nitric oxide (NO)-production bioassay, compound **3** exhibited significant NO inhibitory activity, while compounds **1** and **2** were less potent than **3**.

Cussonia bancoensis Aurev. & Pellegr. (Araliaceae) is a medium-sized tree of 15−25 m in height. It is found mostly in the dense humid forest and is widespread from Ivory Coast to Nigeria.[1] It is used in Nigerian folk medicine for the treatment of dizziness and infertility in women.[1] Previous work on this species has revealed the presence of flavonoids[2] and triterpene saponins.[3] Phytochemical investigations on other *Cussonia* species have described the presence of saponins having oleanolic acid and hederagenin as aglycons and D-glucose, L-rhamnose, L-arabinose, and D-xylose as sugar moieties.[4,5]

In a systematic search for new and/or bioactive metabolites from plant origin we have investigated the methanolic stem bark extract of *C. bancoensis*. We have isolated two new saponins, 3-*O*-(α-L-arabinopyranosyl)-23-hydroxyursolic acid (**1**) and 3-*O*-(*β*-D-glucopyranosyl)-23-hydroxyursolic acid (**2**), along with four known triterpenoids, namely, stigmasterol, ursolic acid, 23-hydroxyursolic acid (**3**), and 3*β*-hydroxylup-20(29)-en-28-oic acid. These compounds were tested for their inhibitory effects on a LPS-stimulated macrophage cell line (Raw 264.7 cells) on nitric oxide (NO) production. NO has diverse physiological roles and also contributes to immune defenses against viruses, bacteria, and other parasites. However, excess production of NO is associated with various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune diseases, chronic inflammatory diseases, and atherosclerosis.[6] Previous studies have revealed the potency of triterpenes and triterpenoidal saponins against cancer cell lines or the inhibition of NO production in LPS-activated macrophage cell lines.[7−9]

The pulverized dried stem bark of *C. bancoensis* was extracted with methanol and concentrated under vacuum to dryness. The water-soluble portion of the methanol extract was successively extracted with ethyl acetate and *n*-butanol. The dried ethyl acetate-soluble fraction was then subjected to repeated column chromatography to afford four known compounds. On the basis of the comparison of proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra as well as direct comparison (co-TLC) with authentic samples, the known compounds were identified as stigmasterol, ursolic acid, 23-hydroxyursolic acid (**3**),[10,11] and 3*β*-hydroxylup-20(29)-en-28-oic acid.[12]



	R_1	R_2
1		OH
2		OH
3	H	OH
4		AcO
5		AcO

Column chromatographic fractionation of the butanol extract gave four main fractions, and repeated column chromatography of the second fraction mainly afforded two saponins, **1** and **2**. The FABMS of **1** (negative-ion mode)

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Table 1. ^{13}C NMR Spectral Data of Compounds **1**−**3** (at 100 MHz in C_5D_5N)

carbon	**1** δ_C mult.	**2** δ_C mult.	**3** δ_C mult.	carbon	**1** δ_C mult.	**2** δ_C mult.	**3** δ_C mult.
1	38.8 t	38.7 t	39.0 t	21	36.8 t	37.4 t	37.0 t
2	28.6 t	28.4 t	26.9 t	22	31.0 t	31.0 t	33.0 t
3	81.8 d	82.1 d	74.2 d	23	64.8 d	64.5 d	64.6 t
4	43.4 s	42.8 s	43.8 s	24	13.6 q	13.3 q	12.7 q
5	48.5 d	48.6 d	48.0 d	25	16.2 q	15.2 q	15.6 q
6	18.1 t	18.3 t	13.3 t	26	17.4 q	17.2 q	17.5 q
7	33.1 t	33.0 t	33.0 t	27	23.8 q	23.6 q	24.2 q
8	39.9 s	39.8 s	40.0 s	28	179.8 s	179.6 s	179.7 s
9	47.4 d	48.5 d	48.5 d	29	17.3 q	17.1 q	17.1 q
10	37.4 s	37.3 s	37.1 s	30	21.3 q	21.0 q	21.1 q
11	24.8 t	23.7 t	23.9 t	sugar moieties	arabinose	glucose	
12	125.6 d	125.3 d	125.6 d				
13	139.4 s	139.5 s	139.2 s	1′	106.4 d	105.9 d	
14	43.4 s	43.2 s	43.0 s	2′	73.8 d	75.9 d	
15	31.0 t	29.8 t	29.0 t	3′	74.6 d	78.7 d	
16	26.0 t	24.6 t	24.5 t	4′	69.5 d	71.8 d	
17	47.4 s	47.2 s	47.0 s	5′	66.8 d	78.4 d	
18	53.5 d	52.1 d	52.2 d	6′	62.9 d		
19	39.7 d	39.1 d	39.2 d				
20	39.4 d	39.2 d	39.0 d				

gave peaks at *m*/*z* 603 $[M - H]^-$ and 471 $[(M - H) - 132]^-$, respectively, suggesting a molecular weight of 604 and the loss of a pentose moiety. Its IR spectra exhibited broad vibration bands at 3480 cm^{-1} (hydroxy groups) and 1700 cm^{-1} (acid group). The EIMS of compound **1** showed ion peaks at *m*/*z* 472, 248, 203, 175, and 133, which indicated that the aglycon is an amyrin derivative having a free carboxylic group at C-17.[13,14] Its ^{13}C NMR spectrum (Table 1) showed signals at δ 125.6 (C-12) and 139.2 (C-13), characteristic for an urs-12-ene derivative,[11] which was further confirmed by its acid hydrolysis to give 23-hydroxyursolic acid (co-TLC with **3**, ^1H NMR and EIMS) and L-arabinose (co-TLC with authentic sample). The sugar moiety was attached to the aglycon at position C-3 since the ^{13}C NMR signal of the above carbon atom appeared at 81.8 ppm in compound **1**, whereas the chemical shift of the same carbon atom appeared at 74.2 ppm in compound **3**.[10,11] The occurrence of a signal at 179.8 ppm showed the presence of a free carboxylic group at C-17. Further signals at 106.6, 73.8, 74.6, 69.5, and 66.8 assigned to C-1′, C-2′, C-3′, C-4′, and C-5′ were in agreement with an arabinopyranosyl moiety.[15,16]

In the ^1H NMR spectrum of **1** the anomeric proton signal appeared at 4.95 ppm as a doublet with the coupling constant of 7.1 Hz, indicating its α-configuration.[17] Acetylation of **1** in the usual manner provided the tetraacetate **4** with a downfield shift of the AB system signals of the two protons corresponding to CH_2OH-23, thus confirming the location of the arabinosyl moiety at C-3 rather than at C-23. Therefore, the structure of compound **1**, which is described here for the first time, was established as 3-*O*-(α-L-arabinopyranosyl)-23-hydroxyursolic acid.

Compound **2** was obtained as a white amorphous powder, and its molecular weight was deduced from its negative HRFABMS as 634. The main peaks observed in the FABMS (negative-ion mode) were at *m*/*z* 633 $[M - H]^-$ and 471 $[(M - H) - 162]^-$, with the latter corresponding to the loss of a hexose moiety. Its EIMS and IR spectra were very close to those of **1**, suggesting that these two compounds have the same aglycon. This was further confirmed on acid hydrolysis, which also afforded 23-hydroxyursolic acid (co-TLC) and D-glucose (co-TLC with authentic sample), instead of arabinose, as was the case with **1**. The ^{13}C NMR spectrum of compound **2** (Table 1) was similar to that of **1** except for the presence of one carbon atom more than that of **1** (δ 60−90 region). The anomeric carbon signal appeared at 105.8 ppm and its proton at 4.94 ppm as a doublet with the coupling constant of 7.2 Hz, indicating its β-configuration.[18] The acetylation of **2** afforded the pentaacetate **5**. The ^1H and ^{13}C NMR data of the six methyl signals (Me-24−27, -29, and -30) of **1** were in agreement with those of the literature values of **3** (the aglycon of **1**) and related compounds.[10] In light of the above findings, the structure of **2** was elucidated as the new compound 3-*O*-(β-D-glucopyranosyl)-23-hydroxyursolic acid.

Table 2. Effect of Compounds Isolated from *Cussonia bancoensis* on NO Production by LPS-Induced Raw 264.7 Macrophages[a]

compound	IC_{50} (μM) NO inhibition	IC_{50} (μM) cytotoxicity for macrophages
1	47.9	124
2	22.1	126
3	2.6	11
ursolic acid	46.9	58.5
L-NIL[b]	18.6	> 200

[a] The culture media were collected for nitrite determination. Results are the mean from four independent experiments. IC_{50} is defined as the concentration that resulted in 50% decrease in NO production. [b] L-NIL (L-N_6-1-iminoethyl)lysine was used as a standard compound.

In the nitric oxide production inhibition experiment (Table 2), 23-hydroxyursolic acid (**3**) exhibited the highest NO inhibitory activity (IC_{50} 2.6 μM) against LPS-induced macrophage cells. It was even more potent than the standard compound (L-NIL, IC_{50} 18.6 μM). Compounds **1** and **2** also showed activity, although they were less potent than **3**. These findings are in agreement with the work of Ryu et al.,[19] who recently reported the in vitro anti-inflammatory effect of other hydroxyursolic acids.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanaco micro-melting point apparatus. Optical rotations were measured on a JASCO DIP-360 digital polarimeter and IR spectra recorded on a JASCO A-302 spectrophotometer. The ^1H NMR spectra (δ ppm, *J* in Hz) were recorded in C_5D_5N or CD_3OD on a Bruker AM-400 spectrometer (400 MHz), while ^{13}C NMR spectra were recorded in the same solvent on an AM-400 instrument at 100 MHz with tetramethylsilane (TMS) as internal standard. EIMS and FABMS were taken on MAT 311A and JMS HX-110 mass spectrometers, respectively. The purity of the samples was

checked on TLC (Si gel, precoated plates, Merck, PF_{254}, 20 × 20 cm, 0.25 mm). Solvents were distilled prior to use.

Plant Material. The stem bark and leaves of *C. bancoensis* were collected in Bafou village (Menoua Division, Western Province of Cameroon) in April 2000. The plant material was identified by Dr. Zapfack Louis of the Department of Plant Biology of the University of Yaounde I. Specimens documenting the collection are deposited at the Cameroon National Herbarium (ref 16896/SRF/CAM).

Extraction and Isolation. The dried and pulverized stem bark of *C. bancoensis* (4 kg) was extracted with MeOH at room temperature for 3 days, and the MeOH extract concentrated to dryness under reduced pressure. The residue obtained (320 g) was suspended in water and extracted with ethyl acetate and *n*-butanol to afford, after evaporation, 120 and 135 g of an EtOAc extract and a *n*-BuOH extract, respectively.

A part of the EtOAc-soluble extract (75 g) was submitted to silica gel column chromatography using hexane−EtOAc mixtures of increasing polarity for elution. A fraction that was eluted with hexane−EtOAc (9:1) yielded stigmasterol (160 mg). Ursolic acid (210 mg) was obtained directly in pure form as white needles [mp 278−280 °C (lit. 280−282 °C, $[\alpha]_D$ +72° ($CHCl_3$)[20]] from the fraction eluted with hexane−EtOAc (8.5:1.5). 23-Hydroxyursolic acid (**3**) and 3β-hydroxylup-20(29)-en-28-oic acid were poorly separated and were obtained as a mixture from the fraction eluted with hexane−EtOAc (3:2). Separation of both substances was achieved by further purification in a silica gel column continuously eluted with the mixture EtOAc−hexane (7:3): Compound **3** [mp 280−282 °C (hexane−EtOAc), (lit. 283−287 °C, $[\alpha]_D^{25}$ +64° (*c* 0.27, MeOH)[10]] and 3β-hydroxylup-20(29)-en-28-oic acid [mp 270 °C (hexane−EtOAc), lit. 263−267 °C (MeOH/$CHCl_3$). $[\alpha]^{21}{}_D$ +88° (*c* 0.075, C_5D_5N)[12]] were then obtained in pure form as white needles, respectively, in the following amounts (7 g and 600 mg).

A part of the butanol-soluble extract (50 g) was subjected to silica gel column chromatography and eluted with the mixtures of CH_2Cl_2−MeOH of increasing polarity. Four main fractions were obtained. The first one afforded, after repeated silica gel column chromatography, eluted with mixtures of CH_2Cl_2−MeOH (4:1 to 3:2), compounds **1** (300 mg) and **2** (200 mg).

3-*O*-(α-L-Arabinopyranosyl)-23-hydroxyursolic acid (1): white amorphous powder from MeOH/CH_2Cl_2; $[\alpha]^{31}{}_D$ +65.2° (*c* 0.046 g/100 mL, MeOH); IR (KBr) ν_{max} 3480, 2990, 1700, 1640, 1450, 1051 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 0.92 (3H, d, J = 6.7 Hz, H-29), 0.94 (3H, d, J = 6.7 Hz, H-30), 0.95 (3H, s, H-24), 0.97 (3H, s, H-25), 1.00 (3×H, s, H-26), 1.20 (3H, s, H-27). 2.60 (IH, d, J = 11.2 Hz, H-18), 3.68 (1H, d, J = 12.1 Hz, H-23A), 3.69 (1H, dd, J = 11.0, 1.6 Hz, H-5A′), 4.00 (1H, dd, J = 8.9, 3.4 Hz, H-3), 4.24 (1H, dd, J = 11.0, 3.0 Hz, H-5B′), 4.26 (1H, m, H-4′), 4.26 (1H, d, J = 12.1 Hz, H-23B), 4.46 (1H, dd, J = 9.0, 7.2 Hz, H-2′), 4.95 (1H, J = 7.2 Hz, H-1′). 5.45 (br t, H-12); ^{13}C NMR, see Table 1; EIMS *m/z* 472 ($C_{30}H_{48}O4$, aglycon), 248, 203, 175; FABMS (negative) *m/z* 603 $[M - H]^-$, 471 $[(M - H) - 132]^-$; HRFABMS (negative) *m/z* 603.8112 $[C_{35}H_{56}O_8 - H]^-$, calcd 603.8143.

3-*O*-(β-D-Glucopyranosyl)-23-hydroxyursolic acid (2): white amorphous powder from MeOH/CH_2Cl_2; $[\alpha]_D^{31}$ +43.4° (*c* 0.046 g/100 mL, MeOH); IR (KBr) ν_{max} 3490, 2990, 1695, 1635, 1450, 1080, 1050 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 0.91 (6H, d, J = 6.7 Hz, H-29 and H-30), 0.95 (3H, s, H-24), 0.96 (3H, s, H-25), 1.04 (3H, s, H-26), 1.16 (3H, s, H-27), 2.62 (1H, d, J = 11.2 Hz, H-18), 3.73 (1H, d, J = 12.3 Hz, H-23A), 3.95 (1H, m, H-5′), 4.05 (1H, dd, J = 8.8, 3.4 Hz, H-3), 4.22 (1H, d, J = 12.3 Hz, H-23B), 4.22 (1H, dd, J = 5.6, 11.4 Hz, H-6A′), 4.23 (1H, dd, J = 8.5, 8.7 Hz, H-4′), 4.25 (1H, dd, J = 8.5, 8.7 Hz, H-3′), 4.35 (1H, dd, J = 7.2, 8.5 Hz, H-2′). 4.45 (1H, dd, J = 2.2, 11.4 Hz, H-6B′), 4.98 (1H, d, J = 7.2 Hz, H-1′), 5.41 (1H, br t, H-12); ^{13}C NMR, see Table 1; FABMS (negative *m/z* 633 $[M - H]^-$, 471 $[(M - H) - 162]^-$; HRFABMS (negative) *m/z* 633.8251 $[(C_{36}H_{58}O_9) - H]^-$, calcd 633.8218.

Acid Hydrolysis of 1 and 2. Saponins **1** and **2** (15 mg each) were refluxed with 4% H_2SO_4 (5 mL) in MeOH for 1 h. Each reaction mixture was then concentrated under reduced pressure to remove MeOH, diluted with H_2O, and filtered. The precipitate of each was purified by recrystallization from MeOH to afford the same aglycon, 23-hydroxyursolic acid, as needles (mp 280−281 °C), which was identified by direct comparison with an authentic sample. Each filtrate was adjusted to pH 7 with $BaCO_3$ and filtered. The two filtrates were concentrated and examined by TLC (MeOH−CH_2Cl_2−AcOH−H_2O, 40:55:3:2). Arabinose from saponin **1** and glucose from saponin **2** were identified by co-TLC comparison with authentic samples.

3-*O*-(α-L-Arabinopyranosyl)-23-hydroxyursolic Acid 23,2′,3′,4′-Tetraacetate (4). A solution of **1** (20 mg) in Ac_2O−pyridine (1:1) was stirred overnight. The reaction mixture was evaporated to dryness and chromatographed on silica gel using hexane−EtOAc (1:1) to give a tetraacetate (**4**) as a white powder in hexane−EtOAc: IR (KBr) ν_{max} 2990, 1740, 1690, 1450, 1380 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 0.80 (3H, s, H-24), 0.82 (3H, s, H-25), 0.94 (3H, d, J = 6.5 Hz, H-29), 0.99 (3H, d, J = 6.5 Hz, H-30), 1.00 (3H, s, H-26), 1.18 (3H, s, H-27), 1.95, 2.01, 2.15, and 2.22 (3H each, s, 4 × OAc), 2.62 (1H, d, J = 11.3 Hz, H-18H), 3.81 (1H, dd, J = 11.8, 4.7 Hz, H-3), 3.91 (1H, dd, J = 13.1, 1.5 Hz, H-5′ax), 4.01 (1H, d, J = 11.3 Hz, H-23A), 4.12 (1H, br d, J = 13.2 Hz, H-5′eq), 4.22 (1H, d, J = 11.2 Hz, H-23B), 4.91 (1H, d, J = 7.5 Hz, H-1′), 5.40 (1H, br t, H-12), 5.52, 5.60, 5.70 (1H, m, H-23, H-2′, H-4′).

3-*O*-(β-D-Glucopyranosyl)-23-hydroxyursolic Acid 23,2′,3′,4′,6′-Pentaacetate. Compound **2** was acetylated and purified as described above to yield a pentaacetate (**5**) as a white powder (hexane/EtOAc): IR (KBr) ν_{max} 2995, 1745, 1690, 1450, 1383 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 0.78 (3H, s, H-24), 0.80 (3H, s, H-25), 0.93 (3H, d, J = 6.2 Hz, H-29), 0.99 (3H, d, J = 6.0 Hz, H-30), 1.02 (3H, s, H-26), 1.20 (3H, s, H-27), 1.98, 1.99, 2.05, 2.12, 2.22 (3H each, s, 5 × OAc), 2.65 (IH, d, J = 11.1 Hz, H-18), 3.96 (1H, dd, J = 12.0, 4.8 Hz, H-3), 3.98 (1H, dd, J = 12.4 Hz, H-23A), 4.32 (IH, d, J = 12.4 Hz, H-23B), 4.08 (1H, dd, J = 12.2, 2.5 Hz, H-6′A), 4.25 (1H, dd, J = 12.2, 4.7 Hz, H-6′B), 5.18, 5.05, 4.93, 3.65 (1H, m, H-2′, H-3′, H-4′, H-5′), 5.38 (1H, br t, H-12).

Bioassays. Cell Culture and Sample Treatment. The Raw 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). DMEM medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies, Inc. (Grand Island, NY). L-N^6-(1-Iminoethyl)lysine (L-NIL) and *E. coli* lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St Louis, MO). These cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2. Cells were incubated with triterpenoids at various concentrations and stimulated with LPS at 1 g/mL for 24 h.

Nitrite Assay. Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by the Griess reaction.[9] Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine HCl] and incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium.

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