Minor Biflavonoids from *Lophira alata* Leaves

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The leaves of the Cameroonian medicinal plant *Lophira alata* afforded two new biflavonoids, lophirone L (**1**) and lophirone M (**2**), and the known luteolin and lithospermoside. Both biflavonoids were obtained in small quantities, and their structures show some new and unusual biflavonoid diversity.

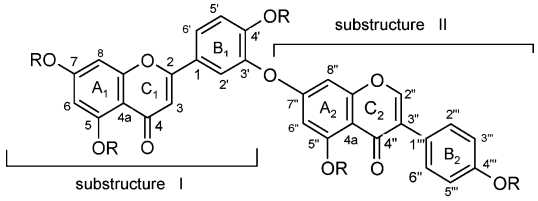
Lophira alata Banks ex Gaertn (Ochnaceae) grows as tall trees in the tropical rain forest of Africa. Its stem bark is exploited for medicinal uses, and its heartwood is sold as first-grade timber. While searching for plant extracts in the Cameroon flora with antitumor activity, the MeOH extract of the stem bark of *L. alata* showed significant suppressive activity on Epstein Barr virus.[1] Some of the polyflavonoids identified in earlier phytochemical investigations show significant antimicrobial activity.[2−9] Our investigation has been extended to the leaf extract (MeOH), from which luteolin and lithospermoside[10] were identified as major constituents. We have now carried out further examination on the same extract obtained using plant material from a different habitat and characterized four constituents. Two are new biflavonoids, for which structures **1** and **2** have been assigned from spectroscopic and chemical data.

The solvent-free MeOH extract of air-dried leaves of *L. alata* was washed with warm EtOAc to give a soluble EtOAc fraction. After concentration to dryness, the resultant powder was fractionated into four fractions (F_1, F_2, F_3 and F_4) by column chromatography on Sephadex LH-20 (MeOH). The purification of fraction F_4 by silica gel column chromatography first gave luteolin and lithospermoside, both identified by comparison with authentic samples. The resultant unseparated subfractions were purified by repeated preparative TLC on silica gel plates. The technique of multiple migration led to the isolation of compounds **1** and **2**. Both gave a brick red color with Mg/HCl and a deep blue color with aqueous $FeCl_3$ solution, suggesting that they are flavonoids.

Compound **1**, obtained as an amorphous yellow solid, is a biflavonoid with molecular mass 538. Its molecular formula, $C_{30}H_{18}O_{10}$, accounting for 22 unsaturation sites, is deduced from its $[M + H]^+$ ion, which appeared at 539.0974 in the HRCIMS. It showed intense IR absorptions indicative of the presence of hydroxyls (3390 and 3250 cm^{-1}), conjugated and hydrogen-bonded carbonyls (1640 and 1634 cm^{-1}), a conjugated double bond (1620 cm^{-1}), and aromatic rings (1605 and1504 cm^{-1}).

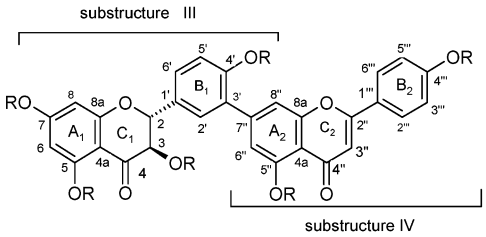
Its ^{13}C NMR spectrum revealed the presence of 30 sp^2-hybridized carbons, including two carbonyls (181.0 and 183.0 ppm); 10 quaternary carbons each substituted by an oxygen atom at 144.8, 149.1, 156.0, 158.9, 159.2, 162.9, 163.1, 164.4, 165.0, and 165.1 ppm; five quaternary carbons at 105.2, 106.0, 122.2, 127.2, and 129.0 ppm; and 13 tertiary carbons at 94.8, 94.9, 99.8, 100.0, 105.1, 116.0, 117.0 (×2), 122.0 (×2), 129.1, 131.0, and 156.1.

Evidence that compound **1** has five phenolic groups came from its complete acetylation (Ac_2O/pyridine), which gave the derivative **1a** ($C_{40}H_{28}O_{15}$), the IR spectrum of which showed no residual OH absorption. Its HRCIMS spectrum displayed the $[M + H]^+$ ion at *m*/*z* 749.1502 (calculated for $C_{40}H_{29}O_{15}$, 749.1506), in agreement with a penta-acetate of lophirone L. The presence of sharp singlets



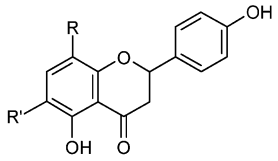
1: R=H Lophirone L

1a: R=Me_2CO Lophirone L penta-acetate



2: R=H Lophirone M

2a R=Me_2CO Lophirone M hexa-acetate



3 : R = OH , R' = H

4 : R = H, R' = OH

for five acetyl methyls at 2.22 (3H, s), 2.33 (3H, s), 2.34 (6H, s), and 2.35 (3H, s) in its ^1H NMR spectrum further confirms the presence of five acetate groups on **1a**.

The ^1H NMR spectra (1D and 2D COSY) clearly showed that the following proton systems are implicated in the structure: one AA′BB′ spin system of four aromatic protons (ring B_2) [δ_H 6.88 (2H, d, 8.9 Hz) and 7.02 (2H, d, 8.9 Hz)]; an ABX spin system of three aromatic protons (ring B_1) [δ_H 6.90 (1H, d, 8.8 Hz), 8.03 (1H, dd, 2.4, 8.8 Hz), and 8.14 (1H, d, 2.4 Hz)]; and two tetrasubstituted aromatic rings each with a pair of *meta*-coupled protons: δ_H 6.25 and 6.53 (both 1H, d, $J = 2.1$ Hz) (ring A_1) and δ_H 6.29 and 6.45 (each 1H, d, $J = 2.1$ Hz) (ring A_2). The two singlets at δ_H 6.75 (1H) and 8.35 (1H) are indicative of the characteristic chemical shifts of H-3 in flavones and H-2 in isoflavones, respectively.

The complete assignments of the protonated carbon resonances were made using the HSQC spectrum of **1**, while a detailed analysis of the HMBC data (Table 1, Supporting Information) led to the definition of two substructures, I and II. Observed cross-peaks indicated connections of the quaternary oxygen-bearing carbon at 165.0 ppm (C-2) and H-3 (δ_H 6.75, δ_C 105.1) and H-2′ (δ_H 8.14, δ_C 131.0, ring B_1), while the C═O at 183.0 ppm was correlated to

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H-3 (δ_H 6.75), clearly suggesting the existence of a flavone moiety as partial structure I. Similarly, connections observed for the quaternary carbon at 122.2 (C-3″) and H-2‴ (δ_H 7.02, δ_C 122.0, ring B_2) and H-2″ (δ_H 8.35, δC-2″ 156.1) and also for the C═O at 181.0 and H-2″ (δ_H 8.35) defined an isoflavonoid moiety as the second partial structure, II. The *peri* OH groups at C-5 (ring A_1) and C-5′ (ring A_2) gave separate singlets at δ 12.92 and 12.82, respectively.

Partial structures I and II can only be linked by an ether bond engaging one of the oxygen substituents. This was confirmed from the NOESY spectrum of **1** in which a strong correlation was observed between H-2′ (δ_H 8.14 ppm, ring B_1) and H-6″ (δ_H 6.29 ppm, ring A_2), leading to a biflavonyl ether with structure **1** for lophirone L.

The EIMS of **1** had intense retro Diels−Alder peaks, giving rise to the base peak at *m*/*z* 152 and the ion at *m*/*z* 386.

It is important to emphasize that **1** is the first report of a biflavonyl ether with mixed flavone−isoflavone substructures from natural sources and also the first report of the existence of a natural biflavonyl ether in the genus *Lophira*. Our results also rule out earlier impressions that biflavonoid ethers are only typical of Asian Ochnaceae.

Compound **2**, a pale yellow, amorphous solid, had an $[M + H]^+$ peak at *m*/*z* 541.1139 in the HRCIMS (541.1135 calculated for $C_{30}H_{21}O_{10}$). This implied a biflavonoid with molecular mass of 540 and molecular formula of $C_{30}H_{20}O_{10}$, accounting for 21 unsaturation sites. Its IR spectrum showed intense absorption bands for hydroxyl groups (3412 and 3250 cm^{-1}), a conjugated and hydrogen-bonded carbonyl (1634 cm^{-1}), a conjugated double bond (1620 cm^{-1}), and aromatic rings (1590 and 1503 cm^{-1}).

Its complete acetylation (Ac_2O/pyridine) gave **2a** ($C_{40}H_{30}O_{16}$), which showed no residual IR hydroxyl absorption. It had an $[M + H]^+$ ion at *m*/*z* 767.1615 in its HRCIMS spectrum, clearly indicating a hexa-acetate (calculated for $C_{40}H_{31}O_{16}$: 767.1612). This was confirmed by six acetyl methyl singlets at δ 2.16, 2.24, 2.26, 2.29, 2.32, and 2.34 in the ^{1}H NMR spectrum of **2a**.

The ^{13}C NMR spectrum of **2** had signals for 30 carbons, among which 28 were sp^2 hybridized. This includes two conjugated and hydrogen-bonded carbonyl carbons at 198.0 and 183.0; eight quaternary carbons each substituted by an oxygen atom at δ 158.6, 159.2, 162.0,164.2, 164.8, 165.1, 165.3, and 167.5; six quaternary carbons at δ 103.0, 106.8, 124.0, 124.1, 128.0, and 130.4; and 12 tertiary carbons at δ 96.0, 97.0, 104.4, 108.3, 110.1, 115.9(×2), 116.7, 128.2, 130.0, and 131.1(×2). Two sp^3 tertiary oxygen-bearing carbon atoms gave signals at δ 83.3 and 85.5. All the chemical shifts of protonated carbons were confirmed using the HSQC spectrum of **2**.

The analysis of the 1D and 2D COSY ^{1}H NMR spectra of **2** revealed that the following proton systems were incorporated in its structure: an AA′BB′ spin system [δ_H 6.74 (2H, d, 8.4 Hz) and 7.30 (2H, d, 8.4 Hz) (ring B_2)]; an ABX aromatic ring system [δ_H 6.96 (1H, d, 9.1 Hz), 7.75 (1H, dd, 2.1, 9.1 Hz), and 7.74 (1H, d, 2.4 Hz) (ring B_1)]; and two sets of isolated *meta*-coupled aromatic protons [δ_H 5.99 and 6.01 (both 1H, d, 1.9 Hz), ring A_1; and δ_H 7.24 and 7.48 (both 1H, d, 1.9 Hz), ring A_2]. An AB system comprising two deshielded aliphatic protons belonging to two oxymethine groups gave doublets at δ_H 4.77 and 5.93 (J = 12 Hz), suggesting the presence of a 3-hydroxyflavanone unit in this structure.

Cross-peaks in the HMBC spectrum of lophirone M (**2**) defined two substructures, designated III and IV (Figure 1). HMBC connections were observed between the carbonyl at δ 198.0 and H-2 (δ_H 5.93) and H-3 (δ_H 4.77). H-2 (δ_H 5.93) was connected to C-2′ (δ_C 131.0), confirming the presence of a 3-hydroxyflavanone moiety as partial structure III. The relative *trans* configuration of H-2 and H-3 was confirmed from the large coupling constant (12 Hz). C-1‴ (δ_C 130.0) was connected to H-3″ (δ_H 6.53), while H-2‴ (δ_H 7.30) was correlated to the carbon C-2″ (δc 164.8), hence leading to partial structure IV. This is a flavone residue, as can be deduced from the characteristic chemical shift of the H-3″ proton (δ_H 6.53). The *peri* OH groups in substructures III and IV appeared as singlets respectively at δ 12.96 and 12.23.

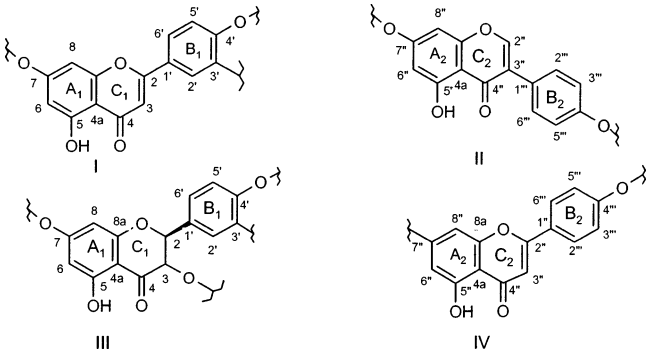
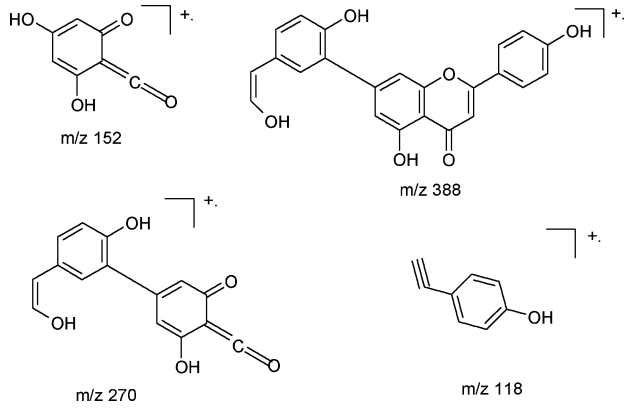


Figure 1. Proton systems and substructures in **1** and **2**.

Scheme 1



Further examination of the HMBC spectrum of **2** clearly showed connections between C-3′ (δ_C 124.1) and both H-8″ (δ_H 7.48) and H-6″ (δ_H 7.24) (Table 1). This unambiguously established the interflavanyl link between C-3′ and C-7″.

The preferential susceptibility of the 3-hydroxyflavanone ring in its EIMS is explained by a retro Diels−Alder fragmentation giving two intense peaks at *m*/*z* 152 (100%) and *m*/*z* 389 (92%). The ionization of the molecule probably involves both flavonoid substructures since ions from a second retro Diels−Alder fragmentation appeared at *m*/*z* 118 and 270. The presence of ions at *m*/*z* 388, 270, and 118 (Scheme 1) further confirms the absence of an oxygenated function at C-7.

The circular dichroism (CD) spectrum of **2** showed a positive Cotton effect at $\lambda_{366\ nm}$ ($\Delta\epsilon = +2.684$) and a negative Cotton effect at $\lambda_{288\ nm}$ ($\Delta\epsilon = -6.968$). Both values implied the absolute configurations 2*R*, 3*R* for the two asymmetric carbons in lophirone M in accordance with literature.[11]

It is important to note the absence of an oxygenated substituent at C-7 in **2**. Generally, flavonoid biosynthesis has always implicated the presence of an oxygen atom at C-7 (ring A), but only very few exceptions such as **3** and **4** have so far been reported in *Artemisia campestris*.[12] These are flavanones with unusual ring A hydroxylation, as they lack the C-7 hydroxyl group. **2** is, thus, the first report of a biflavonoid without an oxygenated function at C−7.

Experimental Section

General Experimental Procedures. IR spectra were recorded using KBr disks on a JASCO FTIR-300E spectrometer. UV spectra were obtained on a Kontron-Uvikon 930 spectrophotometer, and optical rotations were measured on a Perkin-Elmer 341 polarimeter. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained on a Bruker WM 400 spectrometer with Me_2CO-d_6 as solvent and TMS as internal

standard. For HMBC spectra the delay was 70 μs with $J_{CH} \approx 7$ Hz. Sephadex LH-20 (Pharmacia Fine Chemicals) and silica gel 60 (Merck) were used for CC. Solvent used for TLC was a CH_2Cl_2/MeOH mixture (10:1, v/v). Preparative TLC plates on a glass support were coated with fluorescent Si gel (F_{254}). HRESI mass spectra were registered using NH_3 as ionizing gas on an applied biosystems APIQ-STAR PULSAR spectrometer. EIMS were recorded on a Nermag R10-10 apparatus.

Plant Material. *Lophira alata* Banks ex Gaertn (Ochnaceae) leaves were harvested in Mbankomo district near Yaounde, Cameroon, in December 2002. Its identification was made by Mr. Paul Mezili, botanist in the National Herbarium in Yaounde (Cameroon), where a voucher specimen (no. PM 3481) was deposited.

Extraction and Isolation. The air-dried plant material was reduced to a fine powder (1.5 kg) and extracted at room temperature in a percolator, first with CH_2Cl_2 and then with MeOH, to give two extracts. Removal of MeOH from the second extract gave a brown gum (143 g), which was further washed with warm EtOAc. The solvent was removed from the former to give the crude brown EtOAc extract (38 g). Fractionating by CC (Sephadex LH-20 with MeOH as eluent) gave 168 fractions of 50 mL each. They were combined into four fractions: F_1 (23.1 g), F_2 (12.2 g), F_3 (1.4 g), and F_4 (0.3 g).

Further purification of fraction F_4 by CC on a silica gel support with a gradient mixture of CH_2Cl_2/MeOH (from 0% to 20% MeOH) gave 62 fractions of 25 mL each, combined into three subfractions, F_{4a} (124 mg), F_{4b} (30 mg), and F_{4c} (40 mg), from TLC analysis. Fraction F_{4b} was purified in a similar procedure to give luteolin (10 mg) and lithospermoside (14 mg), identified by physical data and by co-chromatography with reference samples.[10]

The purification of the more polar fraction F_{4c} was carried out on preparative TLC silica gel plates developed with a CH_2Cl_2/MeOH (10:1) mixture and applying the multiple migration technique to give compounds **1** (12 mg) and **2** (8 mg).

Lophirone L (1): ($C_{30}H_{18}O_{10}$) yellow, amorphous powder; UV (MeOH) *c* 0.2, λ_{max} (log ϵ) 333(4.45), 288(4.56), 211(4.85); IR (KBr disk) ν cm^{-1} 3390, 3240, 1634, 1605, 1600, 1204, 1441, 1291, 1225, 1080, 981; NMR (^{1}H, 400 MHz and ^{13}C, 100 MHz, see Table 1); MS (70 eV, 110 °C) *m*/*z* (%) 538(30), 444(24), 430(41), 428(71), 402(26), 401(97), 388(30), 386(38), 385(10), 344(44), 277(30), 265(14), 250(16), 236(17), 221(15), 171(8), 152(100); $[M + H]^+$ ion at *m*/*z* 539.0974 (calcd for $C_{30}H_{19}O_{10}$, 539.0978).

Acetylation. Lophirone L (4 mg) was dissolved in a mixture of Ac_2O (1 mL) and dry pyridine (1 mL) in a 5 mL round-bottom flask The well-corked flask was left in an oven at 60 °C for 4 h, after which it was evaporated to dryness under vacuum. The resultant powder was purified by CC on a Sephadex LH-20 support with MeOH as eluent to give lophirone L penta-acetate (2.5 mg) as a white powder: $C_{40}H_{28}O_{15}$, with $[M + H]^+$ at *m*/*z* 749.1502 (calcd for $C_{40}H_{29}O_{15}$); ^{1}H NMR (400 MHz, Me_2CO-d_6) δ 8.39 (1H, s, H-2″),8.25 (2H, $J = 8.5$ Hz, H-3‴, H-5‴), 8.15 (1H, dd, $J = 8.4$, 2.4 Hz, H-6′), 7.50 (2H, d, $J = 8.5$ Hz, H-2‴, H-6‴), 7.19 (1H, d, $J = 8.8$ Hz, H-5′), 7.10 (1H, d, $J = 2.4$ Hz, H-2′), 6.82 (1H, d, $J = 2.2$ Hz, H-8), 6.79 (1H, s, H-2); 6.56 (1H, d, $J = 2.2$ Hz, H-6), 6.51 (1H, d, $J = 2.1$ Hz, H-8″), 6.48 (1H, d, $J = 2.1$ Hz, H-6″), 2.35 (3H, s, CH_3CO); 2.34 (6H, s, 2 CH_3CO); 2.33 (3H, s, CH_3CO); 2.22 (3H, s, CH_3CO).

Lophirone M (2): $C_{30}H_{18}O_{10}$, light yellow, amorphous powder; $[\alpha]_D^{25}$ +13 (MeOH, *c* 0.2); UV (MeOH) λ_{max} (log ϵ) 366(3.56), 288(4.35), 208(4.56); IR (KBr disk) ν cm^{-1} 3412, 3250, 3005, 2935, 2852, 1634, 1590, 1503, 1450, 1361, 1250, 1162, 1096, 806; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz), see Table 1; MS (70 eV, 110 °C) *m*/*z* (%) 540(10), 435(17), 415(21), 414(62), 388(92), 386(71), 384(6), 270(18), 254(10), 234(20), 206(23), 170(20), 152(100), 125(5), 118(12), 106(20), 66(14), 42(3); HRMS (NH_3) $[M + H]^+$ at 541.1434 (calcd for $C_{30}H_{21}O_{10}$, 541.1439).

Acetylation of **2** (5 mg) following the same procedure as above gave after workup 3.1 mg of **2a**. $C_{40}H_{30}O_{16}$: ^{1}H NMR (400 MHz, acetone) δ 7.73 (1H, dd, $J = 8.6$, 2.4 Hz, H-6′), 7.64 (2H, d, $J = 8.5$ Hz, H-2‴, H-6‴), 7.23 (1H, d, $J = 8.6$ Hz, H-5′), 7.21 (1H, d, $J = 2.4$ Hz, H-2′), 7.20 (2H, d, $J = 8.5$ Hz, H-3‴ and H-5‴), 7.19 (1H, d, 2.1 $J =$ Hz, H-6); 7.16 (1H, d, $J = 2.1$ Hz, H-8); 6.59 (1H, s, H-3″); 6.28 (1H, d, $J = 1.8$ Hz, H-8″); 6.23 (1H, d, $J = 1.8$ Hz, H-6″); 5.96 (1H, d, $J =$ 12.5 Hz, H-3); 5.90 (1H, d, $J = 12.5$ Hz, H-2); 2.36 (3H, s, CH_3CO); 2.32 (3H, s, CH_3CO); 2.31 (3H, s, CH_3CO); 2.26 (3H, s, CH_3CO); 2.24 (3H, s, CH_3CO); 2.21 (3H, s, CH_3CO); HRCIMS $[M + H]^+$ at *m*/*z* 767.1615 (calcd for $C_{40}H_{31}O_{16}$, 767.1612).

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Supporting Information Available: NMR 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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