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Two biflavonoids from *Ouratea nigroviolacea*

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Abstract

The leaves of *Ouratea nigroviolacea* (Ochnaceae) afforded two biflavonoids, ouratine A and B together with agathisflavone and stigmasterol. The biflavonoids were characterized as 4′-*O*-methylated apigeninyl-(I-6, II-8)-4′-*O*-methylatedapigenin and 4′-*O*-methylated apigeninyl-(I-6, II-8) apigenin by spectral and chemical transformation studies.

Keywords: *Ouratea nigroviolacea*; Ochnaceae; Biflavonoids; Ouratine A and B

1. Introduction

Ouratea nigroviolacea C. Farron (Ochnaceae) is a slender under shrub found widely throughout the hilly tracts of South Cameroon (Keay, 1989). The genus *Ouratea* is rich in biflavonoids (Moreira et al., 1994; Moreira et al., 1999; Velandia et al., 2002; Felicio et al., 2004; Daniel et al., 2005; Ferreira et al., 2006) and some of the members of this genus are extensively used in traditional Cameroonian medicine (Bouquet, 1969) in the treatment of dysentery, diarrhea, rheumatic and gastric distress. In a continuation of our investigations on biflavonoids of *Ouratea* species (Ngo Mbing et al., 2003; Pegnyemb et al., 2005) we investigated the leaves of *O. nigroviolacea*, a plant hitherto not examined for its chemical constituents. In this study, we report the isolation and characterization of two new biflavonoids together with the known agathisflavone and stigmasterol. The structures of known compounds were determined by analysis of physical and spectroscopic evidence, and confirmed by comparing with the literature data.

2. Results and discussion

The methanolic extract of the leaves of *O. nigroviolacea* was resuspended in MeOH–H_2O (8:2), and then partitioned with EtOAc. The EtOAc extract was fractionated by chromatographic columns to give the new biflavonoids (**1**–**2**) and the known compounds agathisflavone and stigmasterol. The latter two compounds were identified by comparing their physical and spectroscopic data to those reported for agathisflavone (Geiger, 1994) and stigmasterol (Diakow et al., 1978).

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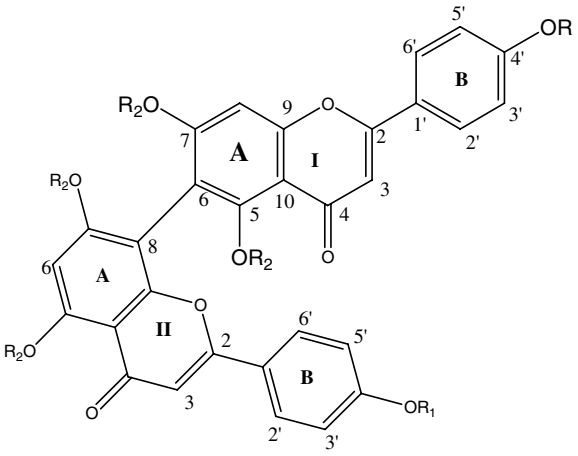

1. R=R_1=CH_3, R_2=H
1a. R=R_1=CH_3, R_2=$COCH_3$
2. R=CH_3, R_1=R_2=H

Ouratine A (**1**) was obtained as yellow crystals from MeOH (m.p. 311–312 °C) and gave a positive reaction with the Neu reagent (Wagner and Bladt, 1996). The molecular formula $C_{32}H_{22}O_{10}$ was deduced from ESI-MS spectrum (*m/z* 567.1, $[M + H]^+$). The IR spectrum exhibited absorption bands at 2928 (—OH), 1652 (chelated —C=O), and bands attributable to aromatic rings at 1575 and 1510 cm^{-1}. The UV spectrum showed absorptions at λ_{max} 329 and 275 nm suggesting the presence of flavone moieties. The ^{13}C NMR spectrum of **1** (Table 1, DMSO-d_6) displayed signals for 32 carbons of the molecule including two sp^3 carbons, two carbonyl carbons at δ 182.5 and 182.3, 16 quaternary sp^2 carbons with eight linked to an oxygen atom and 12 tertiary sp^2 carbons. The ^1H NMR spectrum (Table 1) showed two singlets at δ 13.29 and 13.04 attributed to two H-bonded hydroxyl groups, two sets of A_2B_2-type doublets, one set at δ 8.11 and 7.16 and the other at δ 7.70 and 6.98 attributed to two AA′BB′ systems in two *para*-substituted aromatic rings, four singlets at δ

Table 1
^1H and ^{13}C NMR data for compounds **1**, **2**

Position	DEPT	**1**[a]		DEPT	**2**[b]	
		δC	δH, *J* in Hz		δC	δH, *J* in Hz
2-I	C	163.54		C	165.0	
3-I	CH	103.9	6.88, *s*	CH	105.6	6.77, *s*
4-I	C	182.5		C	183.5	
5-I	C	160.1		C	161.8	
5-OH			13.29, *s*			13.52, *s*
6-I	C	99.7		C	104.6	
7-I	C	162.8		C	162.9	
7-OH			10.79			
8-I	CH	94.0	6.95, *s*	CH	95.0	6.80, *s*
9-I	C	157.2		C	158.6	
10-I	C	104.1		C	104.6	
1′-I	C	123.4		C	123.6	
2′-I	CH	128.8	8.11, *d*, 8.1	CH	129.1	8.11, *d*, 8.0
3′-I	CH	123.5	7.16, *d*, 8.1	CH	124.5	7.19, *d*, 8.0
4′-I	C	163.7		C	164.2	
4′-OCH_3	CH_3	56.0	3.90, *s*	CH_3	56.1	3.97, *s*
5′-I	CH	123.5	7.16, *d*, 8.1	CH	124.5	7.19, *d*, 8.0
6′-I	CH	128.8	8.11, *d*, 8.1	CH	129.1	8.11, *d*, 8.0
2-II	C	163.3		C	164.7	
3-II	CH	103.7	6.75, *s*	CH	105.2	6.67, *s*
4-II	C	182.3		C	183.2	
5-II	C	161.1		C	161.7	
5-OH			13.04, *s*			13.19, *s*
6-II	CH	99.8	6.40, *s*	CH	103.9	6.42, *s*
7-II	C			C	162.9	
7-OH			10.79			
8-II	C	99.2		C	100.1	
9-II	C	155.3		C	156.6	
10-II	C	104.0		C	103.9	
1′-II	C	123.4		C	123.6	
2′-II	CH	128.3	7.70, *d*, 8.2	CH	128.9	7.70, *d*, 8.1
3′-II	CH	123.5	6.98, *d*, 8.2	CH	116.8	6.88, *d*, 8.1
4′-II	C	163.5		C	161.8	
4′-OCH_3 (4′-OH)	CH_3	56.0	3.79, *s*			
5′-II	CH	123.5	6.98, *d*, 8.2	CH	116.8	6.88, *d*, 8.1
6′-II	CH	128.3	7.70, *d*, 8.2	CH	128.9	7.70, *d*, 8.1

[a] DMSO.
[b] Acetone-d_6.

6.95, 6.88, 6.75 and 6.40 attributed to four isolated hydrogens attached to sp^2 carbon atoms. Per-acetylation of **1** gave **1a**, which showed four phenolic acetyl groups, supporting the presence of four phenolic hydroxyl groups in **1**. From detailed analysis of the 1D and 2D NMR data, it was possible to conclude that the first set belonged to the 2′,6′ and 3′,5′ protons of the B(I)-ring and the other set to the 2′,6′ and 3′,5′ protons of the other B(II)-ring. The two singlets at δ 6.88 and 6.75 are characteristic of protons H-3(I), and H-3(II) of the two flavones moieties, respectively. The remaining two singlets at δ 6.95 and 6.40 could be assigned to protons of the two pentasubstituted rings A(I) and A(II). These observations and the selective molecular mass of 566 suggested the compound to be a biapigenin. The fact that the molecule is non-symmetrical and that the A(I) and A(II)-ring protons did not show any coupling, meant that the interflavonoid linkage must be of the agathisflavone type (6(I)–8(II)) (Ofman et al., 1995). The upfield portion of the ^{1}H NMR spectrum showed two methoxy resonances at δ 3.90 and 3.79. H-3′,5′(I) and H-3′,5′(II) appeared at δ 7.16 and 6.98, suggesting that the location of the methoxy substituents is at C-4′(I) and C-4′(II). This was also supported by the compar-

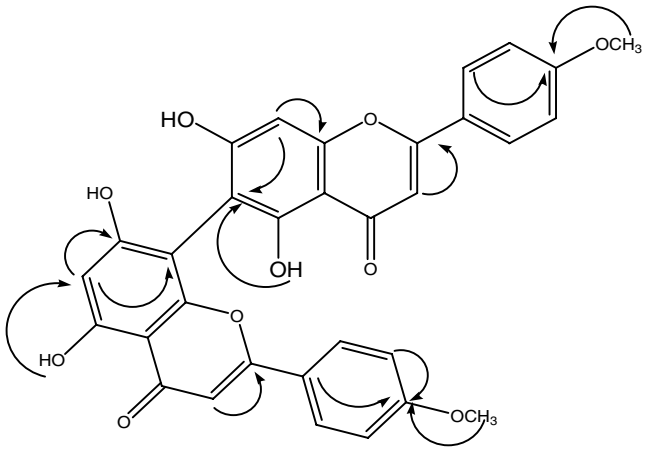

Fig. 1. Key HMBC correlations of **1**.

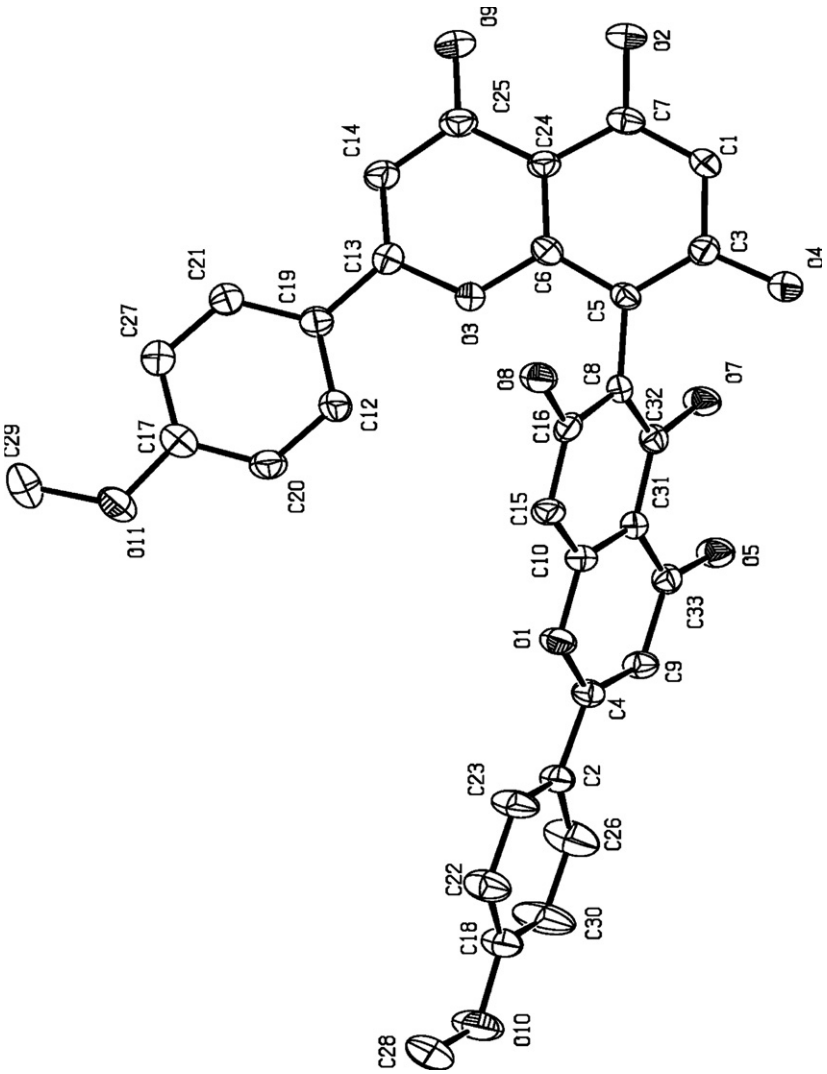

Fig. 2. Ortep representation and the labelling of non-hydrogen atoms (X-ray numbering).

ison of the same protons in agathisflavone 7,7″-dimethylether (Ofman et al., 1995), in which these protons appeared at δ 6.98 (H-3′,5′(I)) and δ 6.77 (H-3′,5′(II)). The HMBC experiment (Fig. 1) of **1** further confirmed the involvement of C-6(I) and C-8(II) in the interflavonoid linkage. H-8 (δ 6.95) was related to C-9 (δ 157.2) and C-6 (δ 99.7) while this latter was also related to 5-OH (δ 13.29). The two methoxyl groups were confirmed to be located in C-4′(I) and C-4′(II) by observing the correlations between their protons and chemical shift assigned carbons of C-4′(I) (δ 163.7) and C-4′(II) (δ 163.5). The structure of **1** was confirmed by a single crystal X-ray analysis. An ORTEP (Spek, 1999) representation of the molecule is shown in Fig. 2. It was thus clearly established as 4′(I),4′(II)-di-*O*-methylagathisflavone (named Ouratine A).

Ouratine B (**2**) was obtained as a yellow powder and was also positive to the Neu reagent (Wagner and Bladt, 1996). The ESI-MS showed a peak corresponding to molecular ion at *m*/*z* 553.1 ($[M+H]^+$, $C_{31}H_{20}O_{10}$), revealing a difference of 14 u between **2** and **1**. The ^1H and ^{13}C NMR, IR and UV spectroscopic data for compounds **1** and **2** were very similar, suggesting that both compounds possessed an identical flavonoid system. The main differences between the ^1H and ^{13}C NMR spectra of **1** and **2** were due to the presence of only one *O*-methyl group (δ 3.97, 56.1) in **2** instead of two *O*-methyl groups in **1**. Slight differences were also found at δ 7.70 (2H, *d*, $J = 8.1$Hz) and 6.88 (2H, *d*, $J = 8.1$ Hz) for the four aromatic protons in ring B(II) of the ^1H NMR spectrum of **2**. These differences suggested a 4′-OH substitution in ring B(II) of **2** instead of a methoxy group in **1** (Geiger and Quinn, 1982; Williams and Fleming, 1995). The HMBC experiment demonstrated that the —OMe protons were related to C-4′(I) (δ 164.2) which was also correlated to H-2′,6′ (δ 8.11). Thus the structure of **2** was established as 4′-*O*-methylagathisflavone (named Ouratine B). The complete assignment of the proton and carbon shifts aided by the comparison with data of **1** is shown in Table 1.

Antimicrobial activities for the CH_2Cl_2/MeOH extract, compounds **1**, **2** and agathisflavone were evaluated using a Mueller–Hinton agar diffusion assay against *Enterococcus sp*., *E. hirae* (ATCC 9790), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus saprophyticus*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* (ATCC 27853) and a range of fungi. No antimicrobial activity was observed for any of the biflavonoids isolated and described here, however the CH_2Cl_2/MeOH extract showed a good activity against *Enterococcus sp*., *E. hirae*, *S. aureus*, *S. saprophyticus*, *A. baumanii*, *P. aeruginosa*.

3. Experimental

3.1. General experimental procedures

Mps were uncorr. U.V. spectra were obtained with a Secoman UV spectrophotometer, while the IR spectra were recorded with a Perkin–Elmer Rx1 FT-IR. ESI-MS experiments were performed on a Micromass Quattro II (altrincham, G.B.). NMR spectra were determined on a Bruker AM 400 WB instrument equipped with a 5 mm ^1H and ^{13}C probe operating at 400 and 100 MHz or a Bruker DPX 200, respectively, with TMS as internal standard.

3.2. Plant material

The leaves of *O. nigroviolacea* were collected at Kribi-Campo, Cameroon, in November 2004 and identified botanically. A voucher specimen (No. 44744/SRF/CAM) is deposited at the National Herbarium in Yaounde, Cameroon.

3.3. Extraction and isolation

The air-dried and powdered leaves (1.37 kg) were extracted with MeOH (15 l) for 3 days at room temperature. The extract was concentrated and dried to give the residue (275 g). Part of this residue (152 g) was resuspended in MeOH–H_2O (8:2). The methanolic aq. layer was defatted with hexane several times (total of 3 l) and then partitioned with EtOAc (3 l). The EtOAc extract (20.4 g) was subjected to normal-phase silica gel column and eluted with CH_2Cl_2–MeOH mixts of increasing polarity (2.5% up to 50%). The fractions were combined into five major fractions on the basis of TLC composition. Fr.2 afforded ppts that were filtered and purified by silica gel column chromatography with 5% MeOH in CH_2Cl_2 to give ouratine A (145 mg), the mother liquors from Fr.2 was submitted on the silica gel column with CH_2Cl_2/MeOH gradient to yield stigmasterol (70 mg). The ppts from fr.3 (86 mg) showed two spots by TLC, and were purified by silica gel with 5% MeOH in CH_2Cl_2 and Sephadex LH-20 using MeOH to give ouratine A (20 mg) and ouratine B (35 mg). Further separation of fr.4 by repeated silica gel, Sephadex LH-20 column chromatography and OPLC gave the last two products and agathisflavone (5 mg). TLC monitoring was performed with C.C. eluents (CH_2Cl_2/MeOH gradient).

3.3.1. Ouratine A (*1*)

Yellow crystals (MeOH), mp 311–312 °C, Rf values: 0.27 (CH_2Cl_2/MeOH 5%), UV (MeOH/CH_2Cl_2) λ_{max}-(log ε): 329 (3.54), 275 (3.51), 222 (3.67), 207 (3.52) nm; IR (KBr) ν_{max}: 2928, 1652, 1606, 1575, 1510, 1459, 1384, 1239, 1182, 836 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6), given in Table 1; ^1H NMR (200 MHz, CD_3COCD_3): δ: 8.12 (2H, *d*, $J = 8.1$ Hz, H-2′, H-6′(I)) 7.78 (2H, *d*, $J = 8.0$ Hz, H-2′, H-6′(II)), 7.20 (2H, *d*, $J = 8.1$ Hz, H-3′and H-5′(I)), 6.98 (2H, *d*, $J = 8.0$ Hz, H-3′ and H-5′(II)), 6.83 (1H, *s*, H-8), 6.79 (1H, *s*, H-3(I)), 6.73 (1H, *s*, H-3(II)), 6.44 (1H, *s*, H-6), 3.97 (3H, *s*, 4′-OCH_3), 3.84 (3H, *s*, 4′-OCH_3); ESI-MS (positive) *m*/*z* 567.1 $[M+H]^+$.

3.3.2. Ouratine A tetraacetate (*1a*)

A mixture of **1** (15 mg), acetic anhydride (2 ml) and pyridine (2 ml) was left to stand overnight at room temperature, and then the solvent was removed. The residue was partitioned between water and CH_2Cl_2. The CH_2Cl_2 extract was subjected to silica gel column chromatography, eluted with 4% MeOH in CH_2Cl_2 to give **1a** (7 mg): ^{1}H NMR (200 MHz, $CDCl_3$): δ: 7.95 (2H, *d*, $J = 7.9$ Hz, H-2′, H-6′(I)), 7.55 (2H, *d*, $J = 7.8$ Hz, H-2′ and H-6′(II)), 7.11 (2H, *d*, $J = 7.9$ Hz, H-3′, H-5′(I)), 7.07 (1H, *s*, H-8), 6.88 (2H, d, $J = 7.8$ Hz, H-3′, H-5′(II)), 6.78 (1H, *s*, H-3(I)), 6.69 (1H, *s*, H-3(II)), 6.62 (1H, *s*, H-6), 3.95 (3H, *s*, 4′-OCH_3), 3.83 (3H, *s*, 4′-OCH_3), 2.13, 2.16, 2,21, 2.50, (each 3H, *s*, $OCOCH_3 \times 4$).

3.3.3. Ouratine B (*2*)

Yellow powder; Rf values: 0.13 (CH_2Cl_2/MeOH 5%); UV (MeOH) $\lambda_{max}(\log\varepsilon)$: 330 (3.50), 274 (3.51) 223 (3.63), 204 (3.60) nm; IR (KBr) ν_{max}: 3005, 1651, 1605, 1574, 1510, 1455, 1354, 1243, 1179, 833 cm^{-1}; ^{1}H NMR (400 MHz, CD_3COCD_3) and ^{13}C NMR (100 MHz, CD_3COCD_3), given in Table 1; ESI-MS (positive) *m/z* 553.12 $[M + H]^+$.

3.4. X-ray diffraction studies

Crystals of **1** were grown by slow evaporation of methanol/chloroform solution at 293 K. The crystal used for X-ray measurement was lamellar, with dimensions: $0.25 \times 0.16 \times 0.12$ mm. Ouratine A, $C_{32}H_{22}O_{10}$, $M_x = 566.50$ g mol^{-1} crystallizes in the orthorhombic system, space group $P2_12_12_1$ ($Z = 4$). The unit cell parameters are as follow: $a = 10.0313(9)$ Å, $b = 14.78874(14)$ Å and $c = 17.9853(16)$ Å with a cell volume of 2668.1(4) Å^3. The calculated density equal to 1.41 g cm^{-3}. The linear absorption coefficient is $\mu = 0.106$ mm^{-1} for the λ(Mo K_α) radiation ($\lambda = 0.71073$ Å). The diffracted intensities were collected with a ENRAF NONIUS Kappa CCD diffractometer. The structure was solved by Direct Methods (SHELXS 97) and refined by full-matrix least squares against F^2 (SHELXL 97) (Sheldrick, 1997). Scattering factors were taken from the International Tables for Crystallography. All non-H atoms were refined anisotropically and the H-atoms were fixed in calculated positions at parent C and O atoms, respectively. The final reliability factors are: $R = 0.0441$, $wR = 0.0830$ [$I > 2\sigma(I)$] and the goodness of fit on F^2 was equal to 0.852. The minimum and maximum residual densities were equal −0.130 and 0.126 e Å^{-3}, respectively.

4. Supplementary material

Crystallographic data for the structure reported in this paper will be deposit at Cambridge Crystallographic Data Centre. These data can be obtained free of charge from the CCDC 600790 via www.ccdc.cam.ac.uk/data_request/cif.

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