Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 1922–1926
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Antimicrobial biflavonoids from the aerial parts of *Ouratea sulcata*

Dieudonné Emmanuel Pegnyemb [a,*], Josephine Ngo Mbing [a,b], Alex de Théodore Atchadé [a], Raphael Ghogomu Tih [a], Beiban Lucas Sondengam [a], Alain Blond [c], Bernard Bodo [c]

[a] *Department of Organic Chemistry, Faculty of Science, University of Yaounde I, P.O. Box 812 Yaounde, Cameroon*
[b] *Centre de Recherches en Plantes Médicinales et Médecine Traditionnelle, I.M.P.M, B.P. 6163 Yaoundé, Cameroon*
[c] *Laboratoire de Chimie et Biochimie des Substances Naturelles, USM 0502 MNHN-UMR 5154 CNRS, 63 rue Buffon, 75005 Paris, France*

Received 1 March 2005; received in revised form 11 June 2005; accepted 20 June 2005
Available online 3 August 2005

Abstract

Investigation of the aerial parts of *Ouratea sulcata* led to the isolation of a biflavonoid named sulcatone A, together with the known compounds, 3-hydroxy-2,3-dihydroapigenyl-[I-4′,O,II-3′]-dihydrokaempferol, amentoflavone, lophirone A, agathisflavone, stigmasterol and stigmasteryl-3-*O*-β-D-glucopyranoside. The structure of the compound was assigned as apigenyl-[I-4′,O,II-3′]-dihydrokaempferol, by means of spectroscopic analysis. Sulcatone A and 3-hydroxy-2,3-dihydroapigenyl-[I-4′,O,II-3′]-dihydrokaempferol exhibited significant in vitro antimicrobial activities against a range of microorganisms.

Keywords: *Ouratea sulcata*; Ochnaceae; Biflavonoids; Sulcatone A and antimicrobial activities

1. Introduction

Ouratea sulcata Van Tiegh (ex Keay), which belongs to the plant family Ochnaceae is widely distributed in West and Central Africa (Keay, 1989). Extracts of leaves, alone or combined with other plants, are used in many African countries, including Cameroon, Nigeria, Congo and Gabon to treat human ailments such as upper respiratory tract infections, dysentery, diarrhoea and toothache (Bouquet, 1969). Plants in this family are known to be rich in biflavonoids (Pegnyemb et al., 2001, 2003; Messanga et al., 1994; Moreira et al., 1999; Tang et al., 2003; Rao et al., 1997; Ngo Mbing et al., 2003). No phytochemical study has been reported for *O. sulcata*. In our continuing investigation of new biologically active metabolites from the Ochnaceae family, we here report the isolation, structural elucidation and biological activities of sulcatone A, as well as six known compounds, 3-hydroxy-2,3-dihydroapigenyl-[I-4′,O,II-3′]-dihydrokaempferol, amentoflavone, lophirone A, agathisflavone, stigmasterol and stigmasteryl-3-*O*-β-D-glucopyranoside.

2. Results and discussion

Air dried leaves of *O. sulcata* were extracted for 48 h with MeOH at room temperature and afterwards filtered and concentrated under vacuum. The crude MeOH extract was re-extracted with CH_2Cl_2/MeOH (1:1) and fractionation of this residue by silica gel column chromatography, afforded a series of active fractions which afforded the new biflavonoid, sulcatone A (**1**) together with six known compounds (**2**–**7**). The latter group of

* Corresponding author. Tel.: + 237 7804176; fax: + 237 2221873.
E-mail addresses: epegny@uycdc.uninet.cm, pegnyemb@yahoo.com (D.E. Pegnyemb).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.06.017

compounds were identified by comparison of their spectroscopic data with literature data as 3-hydroxy-2,3-dihydroapigenyl-[I-4′,O,II-3′]-dihydrokaempferol (**2**) (Sievers et al., 1992), amentoflavone (**3**) (Chari et al., 1977), lophirone A (**4**) (Ghogomu et al., 1987), agathisflavone (**5**) (Geiger, 1994), stigmasterol (**6**) (Diakow et al., 1978) and stigmasteryl-3-*O*-β-D-glucopyranoside (**7**) (Berry et al., 1962), respectively.

Although, **2** is a known compound which has been previously reported from *Hypnum cupressiforme*, it is the first time that it has been isolated from the Ochnaceae family. Its ^{13}C NMR data, which have been assigned on the basis of Jmod, HSQC and HMBC experiments, are reported here for the first time.

Sulcatone A (**1**) was obtained as pale yellow amorphous solid and gave positive reaction to methanolic ferric chloride reagent. Its molecular formula was shown to be $C_{30}H_{20}0_{11}$ from high resolution ESI-TOF mass spectrum which showed a quasimolecular ion $[M+H]^+$ at *m*/*z* 557.0965 (calc. 557.0961). The IR spectrum of **1** disclosed vibration bands due to hydrogen bonded hydroxyl (3242 cm^{-1}), carbonyl (1648 cm^{-1}) and benzene ring moieties. The ^1H NMR spectrum of **1** (Table 1) showed an olefinic signal at δ_H 6.68 (1H, *s*, H-3), characteristic of a flavone structure and two aliphatic protons at δ_H 4.62 (1H, *d*, $J = 11.8$ Hz) and 5.11 (1H, *d*, $J = 11.8$ Hz) typical of a dihydroflavonol nucleus (Foo, 1987). The ^{13}C NMR and DEPT spectra indicated a flavone – dihydroflavonol based biflavonoid structure for **1**, with 30 carbon resonances representing two carbonyls, two aliphatic and twenty six olefinic and aromatic carbons. The flavone unit of **1** appeared to be 5,7,4′-trioxygenated (apigenin) as indicated by signals at δ_H 6.24 (1H, *d*, $J = 2.4$ Hz, H-6); 6.53 (1H, *d*, $J = 2.4$ Hz, H-8); 7.08 (2H, *d*, $J = 8.8$ Hz, H-3′ and 5′); 7.79 (2H, *d*, $J = 8.8$ Hz, H-2′ and 6′), whilst the dihydroflavonol moiety seemed to be tetra oxygenated with five aromatic protons resonating at δ_H 5.98 (1H, *d*, $J = 2.2$ Hz, H-6); 6.02 (1H, *d*, $J = 2.2$ Hz, H-8); 7.16 (1H, *d*, $J = 8.6$ Hz, H-5′); 7.38 (1H, *dd*, $J = 2.2$, 8.6 Hz, H- 6′) and 7.36 (1H, *d*, $J = 2.2$ Hz, H-2′) reminiscent of a 5,7,3′,4′-tetrahydroxydihydroflavonol

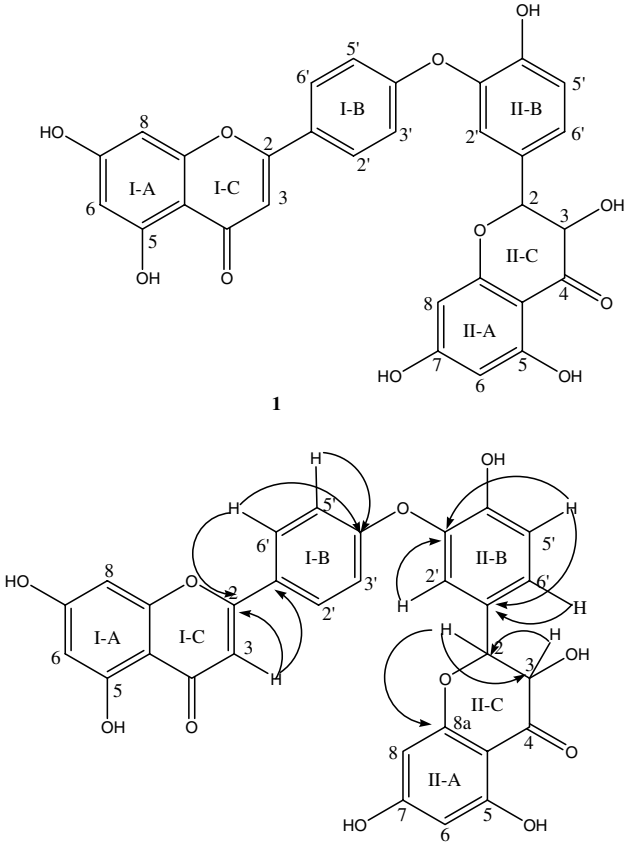
Table 1
^{13}C and ^1H NMR data of compounds (**1**) and (**2**) (acetone-d_6)

No.	(**1**)			(**2**)		
	^{13}C	^1H [*m*, *J* (Hz)]	HMBC	^{13}C (*m*)	^1H [*m*, *J* (Hz)]	HMBC
2	83.7	5.11 (*d*, 11.8)	2′,6′-II-B, 3	83.8	5.14 (*d*, 11.8)	2′,6′-II-B, 3
3	73.0	4.62 (*d*, 11.8)	2	73.0	4.62 (*d*, 11.8)	2
4	197.6	–	3	197.9	–	3
4a	101.5	–	6-II-A	101.4	–	6-II-A
5	164.7		6-II-A	164.7		6-II-A
6[a]	97.2	5.98 (*d*, 2.2)	8-II-A	97.0	5.92 (*d*, 2.4)	8-II-A
7	168.2		6,8-II-A	167.7		6,8-II-A
8[a]	96.0	6.02 (*d*, 2.4)	6-II-A	96.0	5.96 (*d*, 2.4)	6-II-A
8a	164.0	–	8-II-A, 2	164.0	–	8-II-A, 2
1′	130.4	–	2′,6′-II-B, 2	130.4	–	2′,6′-II-B, 2
2′	122.4	7.36 (*d*, 2.2)	6′-II-B	122.3	7.30 (*d*, 2.2)	6′-II-B
3′	142.3	–	2′,5′-II-B	143.2	–	2′,5′-II-B
4′	150.6		2′,5′,6′-II-B	150.6		2′,5′,6′-II-B
5′	118.0	7.16 (*d*, 8.6)	6′-II-B	117.8	7.13 (*d*, 8.8)	6′-II-B
6′	126.9	7.38 (*dd*, 2.2; 8.6)	2′,5′-II-B	126.3	7.31 (*dd*, 2.2; 8.8)	2′,5′-II-B
2	164.7	–	2,6′,3′-I-B	83.9	5.14 (*d*, 11.8)	2,6′,3′-I-B
3	105.2	6.68 (*s*)	2	73.1	4.62 (*d*, 11.9)	2
4	182.9	–	3	197.9	–	3
4a	105.0	–	6-I-A	101.4	–	6-I-A
5	163.7		6-I-A	164.7		6-I-A
6[b]	99.7	6.24 (*d*, 2.4)	8-I-A	97.0	5.92 (*d*, 2.4)	8-I-A
7	165.1		6-8-I-A	167.7		6-8-I-A
8[b]	94.8	6.53 (*d*, 2.4)	6-I-A	96.0	5.96 (*d*, 2.4)	6-I-A
8a	158.8	–	6-I-A, 2	164.0	–	6-I-A, 2
1′	130.5	–	2′,6′-I-B	132.1	–	2′,6′-I-B
2′	129.1	7.79 (*d*, 8.8)	3′,6′-I-B	130.3	7.56 (*d*, 9.0)	3′,6′-I-B
3′	117.4	7.08 (*d*, 8.8)	2′,5′-I-B	116.9	7.01 (*d*, 9.0)	2′,5′-I-B
4′	162.1	–	3′,5′-I-B	159.4	–	3′,5′-I-B
5′	117.4	7.08 (*d*, 8.8)	3′,6′-I-B	116.9	7.01 (*d*, 9.0)	3′,6′-I-B
6′	129.1	7.79 (*d*, 8.8)	2′,5′-I-B	130.3	7.56 (*d*, 9.0)	2′,5′-I-B
OH-4′	–	8.78 *s*			8.79 *s*	
OH-7	–	9.63 *s*			–	

[a,b] Values may be reversed.

(dihydrokampferol, Markham and Geiger, 1994). Further supporting evidence (Table 1) came from ^{13}C NMR data which showed a close resemblance with those of apigenin and dihydrokampferol (Agrawal et al., 1989). Compound **1** is thus a biflavonoid comprising an apigenin unit linked to a dihydrokampferol unit through a ether bond. This hypothesis was confirmed by the C–H correlations observed in the HMBC spectrum (Fig. 1): between H-2′ and C-2 while in the dihydroflavonol part, an HMBC connectivity was found between H-2 and C-2′ and C-6′, and between H-3 and C-2. Thus, the structure of **1** was established as apigenyl-[I-4′,O,II-3′]-dihydrokaempferol named sulcatone A (**1**), the first biflavonoid with flavone and dihydroflavonol units coupled through the B-ring.

Compounds **1** and **2** were tested for their antimicrobial activity against Gram positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio anguillarium*) and Gram negative (*Escherichia coli*) bacteria in an agar well diffusion assay. As shown in Table 2, with all the three tested Gram positive strains (*S. aureus*, *B. subtilis*, *V. anguillarium*) the activities of the compounds **1** and **2** were almost equivalent to or less than those demonstrated by streptomycin. None of these compounds was active (MIC > 100) against Gram negative bacterium, *E. coli*.



1

Fig. 1. Major HMBC correlations observed in **1**.

Table 2
In vitro antimicrobial activity of *O. sulcata* crude extract and compounds **1** and **2**

Compound	Microorganism tested[b]			
	S. aureus	*B. subtilis*	*V. anguillarium*	*E. coli*
MeOH extract	600	600	600	i[+a]
CH_2Cl_2/MeOH (1:1) extract	145	145	145	i[+a]
Compound **1**	12.5	08.12	–	i[+a]
Compound **2**	08.51	10.05	i[+a]	i[+a]
Streptomycin sulfate	06.25	00.85	04.12	i[+a]

[a] In active.
[b] The data are represented as minimum inhibitory concentration (MIC in μg/mL) that prevented growth of microorganism.

3. Experimental

3.1. General experimental procedures

Melting points were measured on a Buchi apparatus and are uncorrected. IR spectra were measured on a JASCO FTIR–300E spectrometer with KBr pellets. UV spectra were recorded on a Kontron–Uvikon 932 spectrophotometer. Optical rotations were measured on a Perkin–Elmer 341 polarimeter. NMR spectra were run on a Bruker instrument equipped with a 5 mm ^1H and ^{13}C probe operating at 400 and 100 MHz, respectively, with TMS as internal standard. ^1H assignments were made using 2D-COSY and NOESY (mixing time 500 ms) while ^{13}C assignments were made using 2D-HSQC and HMBC experiments. Silica gel, 70–230 mesh (Merk) were used for column chromatography, while precoated aluminium sheets silica gel 60 F_{254} were used for TLC. The HR-ESI mass spectra were run on an applied Biosystems API Q-STAR PULSAR. The solvent mixtures used for both CC and TLC were CH_2Cl_2–MeOH (10:1 and 5:1) unless stated otherwise.

3.2. Plant material

Fresh leaves of *O. sulcata* were harvested in November 2002 at Kribi (Cameroon). A voucher specimen (No. 10133/SRF/CAM) documenting the collection was identified at the National Herbarium, Yaounde, Cameroon, and is on deposit there.

3.3. Extraction and isolation

Sun-dried, ground plant material (5 kg) was extracted with cold MeOH in an iron tank equipped with a mechanical stirrer. The crude extract was concentrated to dryness leaving a dark green residue (230 g) that was re-extracted with CH_2Cl_2/MeOH (1:1) to yield a brown extract (72 g). These two extracts were evaluated in vitro for their antimicrobial activity and potency

against a range of representative Gram positive (*S. aureus*, *B. subtilis*, *V. anguillarium*) and Gram negative (*E. coli*) bacteria. As a result, MIC values of the CH_2Cl_2/MeOH and MeOH extracts were 145 and 600 μg/mL, respectively when tested against Gram positive representative. However, no activity was observed against *E. coli* taken as a Gram negative representative. The crude CH_2Cl_2/MeOH (1:1) extract (72 g) was fractionated by column chromatography on silica gel using a gradient of CH_2Cl_2/MeOH, starting from 50:1 to pure MeOH. The fractions were combined into five major fractions (OS_1–OS_5) on the basis of TLC composition. Chromatography of the fraction OS_2 (13.4 g) on a silica gel column with CH_2Cl_2/MeOH gradient yielded compound (**6**) (700 mg). Fraction OS_3 (6.2 g) crystallized in acetone and was applied to a Sephadex LH-20 column with MeOH as eluent, to give amentoflavone (**3**) (600 mg). Fraction OS_4 (7.1 g) was further fractionated by CC into three parts (OS4a:0.8 g; OS4b: 1.8 g; OS4c: 2.9 g). Fractionation of OS4b on a silica gel column with CH_2Cl_2/MeOH (15:1) afforded lophirone A (**4**) (800 mg) and agathisflavone (**5**) (123 mg). Gel filtration of OS4c with MeOH gave amentoflavone (**3**) (400 mg) and a fraction containing mainly stigmasteryl-3-*O*-β-D-glucopyranoside (**7**) (450 mg). OS_5 (28.3 g) was subjected to repeated chromatography on Sephadex LH-20 (MeOH) and preparative TLC (Silica gel, CH_2Cl_2/MeOH, 10:1) to yield two compounds, sulcatone A (**1**) (28 mg) and 3-hydroxy-2,3-dihydroapigenyl-[I-4′,O,II-3′]-dihydrokaempferol (**2**) (32 mg).

3.3.1. Sulcatone A (*1*) $C_{30}H_{20}O_{11}$

Amorphous pale yellow solid; $[\alpha]_D^{25} - 26.5$(MeOH, *c*0.1); UV_{max}^{MeOH}nm(log ε): 332 (4.28), 292 (4.76), 209 (4.48); IR (KBr) ν_{max}: 3168, 1648, 1584, 1536, 1512, 1440, 1316, 1168, 1089, 838 cm^{-1}. ^1H NMR (acetone-d_6) see Table 1, ^{13}C-NMR (acetone-d_6) see Table 1; HRESI-TOF *m*/*z* 557.0965 $[M + H]^+$ (calc. for $C_{30}H_{20}O_{11}$, 557.0961).

3.4. Antimicrobial assay

The extract and purified active flavonoids from *O. sulcata* were tested against, *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633), *V. anguillarum* (ATCC 19264) and *E. coli*. (ATCC 8739). The qualitative antimicrobial assay employed was a classical disc diffusion procedure using Mueller–Hinton agar (DIFCO). Paper discs were impregnated with 20 μL of DMSO solution of each sample (1 mg/mL) and allowed to evaporate at room temperature. The plates with micro-organisms were incubated at 37 °C for 24 h for *S. aureus* and at 27 °C for 48 h for *V. anguillarum*. The diameter of inhibition zone around each disc was measured and recorded at the end of the incubating period. As the sensitivity of the disc bioassay is low, final activity was performed using a minimum inhibitory concentration (MIC) method. The MIC values were determined by the standard broth micro-dilution method in Mueller–Hinton, with an inoculum of 10^4 CFU/mL. To ensure that the densities of the diluted cultures were within the range, serial dilution plate counts were also made for each culture. Tube inoculated with *Bacillus subtilis* were incubated at 30 °C while tubes inoculated with others microorganisms were incubated at 37 °C. The MIC values were determined after 24 h of incubation. Streptomycin sulfate was used as positive control.

Acknowledgements

This research was supported by the International Foundation for Science (IFS), Stockholm, Sweden, and the Organization for Prohibition of Chemical Weapons (OPCW), The Hague, The Netherlands, through a grant to Dr. Pegnyemb No. F/3330-1. We acknowledge Mr. Nana (National Herbarium Cameroon) for his assistance in the collection and identification of the plant material, Dr. J.P. Brouard and Mr. L. Dubost for the mass spectra and Mr. G. Gastine and Dr. Gangoué for antimicrobial screening. The authors are also indebted to the University of Yaounde I grant committee and the French ''Ministère de l'Education Nationale'' for financial assistance.

References

Agrawal, P.K., Thakur, R.S., Bansal, M.C., 1989. In: P.K., Agrawal (Ed.), Carbon 13 NMR of Flavonoids. Elsevier, Amsterdam, pp. 95–182.

Berry, R.C., Burton, W.W., Wright, H.E., 1962. Identification of stigmasteryl-D-glucoside in aged burley tobacco. Journal of Organic Chemistry 27, 918–920.

Bouquet, A., 1969. Féticheurs et Médecines Traditionnelles du Congo Brazzaville. ORSTOM, Paris, pp. 178–179.

Chari, V.M., Ilyas, M., Wagner, H., Nezmelyi, A., Chen, L., Lin, Y., 1977. ^{13}C–NMR spectrocopy of biflavonoids. Phytochemistry 16, 1273–1278.

Diakow, P.R.P., Holland, H.L., Taylor, G.L., 1978. ^{13}C nuclear magnetic resonance spectra of some C-19-hydroxy, C-5,6-epoxy, C-24-ethyl and C-19-norsteroids. Canadian Journal of Chemistry 56, 3121–3127.

Foo, L.Y., 1987. Configuration and conformation of dihydroflavonols from *Acacia melanoxylon*. Phytochemistry 26, 813–817.

Geiger, H., 1994. Biflavonoids and triflavonoids. In: Harborne, J.B. (Ed.), The Flavonoids: Advances in Research Since 1986. Chapman & Hall, London, pp. 95–115.

Ghogomu, T.R., Sondengam, B.L., Martin, M.T., Bodo, B., 1987. Lophirone A, a biflavonoid with unusual skeleton from *Lophira lanceolata*. Tetrahedron Letters 28, 2967–2969.

Keay, R.W.J., 1989. Trees of Nigeria. Clarendon Press, Oxford, UK.

Markham, K.R., Geiger, H., 1994. ^1H nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in hexadeuterodimethylsulfoxide. In: Harborne, J.B. (Ed.), The Flavonoids: Advances in Research since 1986. Chapman & Hall, London, pp. 441–497.

Messanga, B.B., Ghogomu, T.R., Sondengam, B.L., Martin, M.T., Bodo, B., 1994. Biflavonoids from *Ochna calendendron*. Phytochemistry 35, 791–794.

Moreira, I.C., De Carvalho, M.G., Bastos, A.B.F.O., Braz-Filho, R., 1999. A flavone dimer from *Ouratea hexasperma*. Phytochemistry 51, 833–838.

Ngo Mbing, J., Pegnyemb, D.E., Ghogomu, T.R., Sondengam, B.L., Blond, A., Bodo, B., 2003. Two biflavonoids from *Ouratea flava*. Phytochemistry 63, 632–634.

Pegnyemb, D.E., Ghogomu, T.R., Sondengam, B.L., Blond, A., Bodo, B., 2001. Biflavonoids from *Ochna afzelii*. Phytochemistry 57, 579–582.

Pegnyemb, D.E., Ghogomu, T.R., Sondengam, B.L., Blond, A., Bodo, B., 2003. Flavonoids of *Ochna afzelii*. Phytochemistry 64, 661–665.

Rao, K.V., Sreeramulu, K., Rao, C.V., Gunasekar, D., Martin, M.T., Bodo, B., 1997. Two new biflavonoids from *Ochna obtusata*. Journal of Natural Products 60, 632–634.

Sievers, H., Burhardt, G., Becker, H., Zinsmeister, H.D., 1992. Hypnogenols and other dihydroflavonols from the moss *Hypnum cupressiforme*. Phytochemistry 31, 3233–3237.

Tang, S., Bremer, P., Kortenkamp, A., Schlage, C., Gray, A.I., Gibbons, S., Heinrich, M., 2003. Biflavonoids with cytotoxic and antibacterial activity from *Ochna macrocalyx*. Planta Medica 69, 247–253.