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NEOFLAVONOIDS FROM THE STEM BARK OF *CALOPHYLLUM VERTICILLATUM*

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Key Word Index—*Calophyllum verticillatum*; Guttiferae; stem bark; neoflavonoids; caloverticillic acids A, B and C; triterpenoids; molluscicides.

Abstract—The chemical investigation of the extractives of *Calophyllum verticillatum* provided three new neoflavonoids: caloverticillic acids A, B and C, in addition to α-amyrin and friedelin. Molluscicidal activity against *Biomphalaria glabrata* was observed.

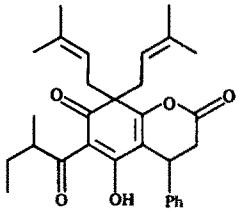
INTRODUCTION

Numerous *Calophyllum* species (Guttiferae) are recorded in Madagascar and the chemical investigation of several *Calophyllum* species: *C. inophyllum* L. [1, 2], *C. recedens* Jumelle Barrier (3), *C. chapelieri* Drake [4], has been reported. The seeds of another Madagascan endemic, *C. verticillatum* have been shown to contain calofloride (**1**), a neoflavonoid of a new structural type [5]. Since calofloride showed interesting molluscicidal activity [6] we decided to investigate other organs of *C. verticillatum* and a chemical analysis of the bark led to the isolation of two chemically distinct groups of compounds: neoflavonoids and triterpenoids.

RESULTS AND DISCUSSION

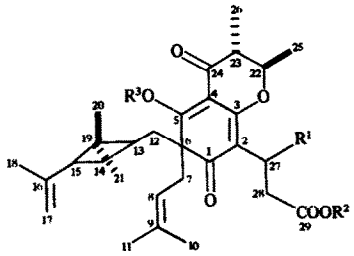
A methylenechloride extract of the finely ground bark of *C. verticillatum* was chromatographed over silicic acid. Elution with petrol–ethylacetate gave first a crude mixture of triterpenoids which on further purification afforded friedelin [7] and α-amyrin [8].

The more polar neoflavonoids were eluted with petrol–ethyl acetate. Repeated chromatography of this mixture provided a major fraction whose physical data indicated a close relationship with well known characteristic Guttiferae constituents such as the complex acids extracted by Stout *et al.* from the bark resins of *C. brasiliense* and *C. inophyllum* [9, 10]. Indeed, a strongly chelated phenolic hydroxyl group could be easily detected by spraying the TLC plates with alcoholic ferric chloride.

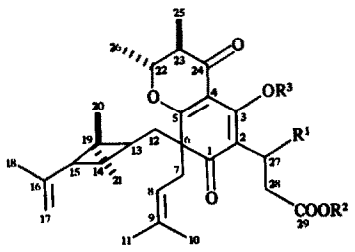


1 Calofloride

Its presence was further confirmed by a deshielded proton signal at about δ15 ppm in the ^{1}H NMR spectrum. The final identification of the new neoflavonoid acids of *C. verticillatum* was made as their more stable methoxymethylesters, which were obtained by treating the crude mixture with diazomethane in methanol. Further



	R¹	R²	R³
2	*n*-pentyl	Me	Me
2a	*n*-pentyl	H	H
3	*n*-pentyl	Me	Me
3a	*n*-pentyl	H	H
5	*n*-propyl	H	H



	R¹	R²	R³
4	*n*-pentyl	Me	Me
4a	*n*-pentyl	H	H
6	*n*-propyl	H	H

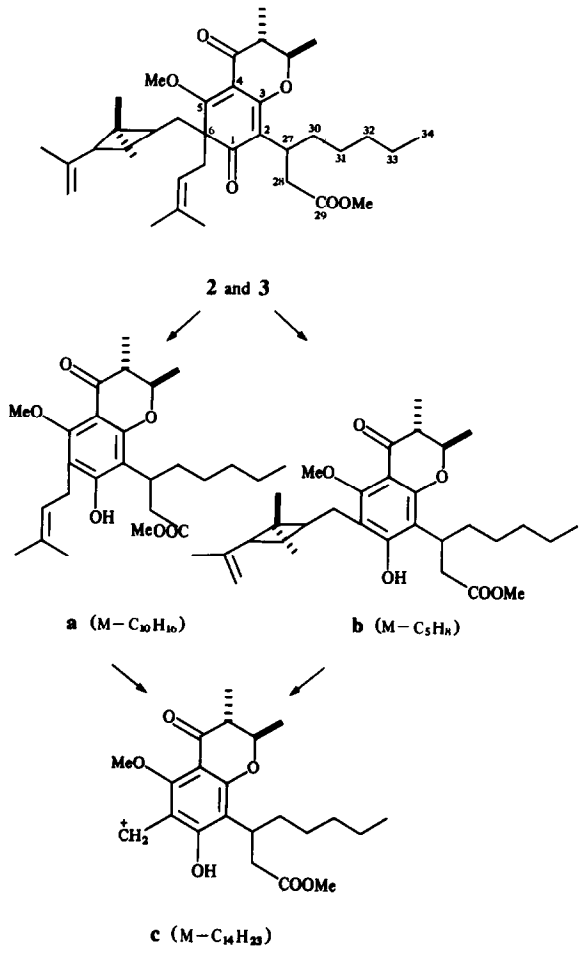


Fig. 1.

chromatographic purification led to the isolation of three isomeric methoxymethylesters **2**, **3** and **4**, as confirmed by high resolution analysis of their molecular ion peak ($C_{36}H_{54}O_6$). The mass spectra of all three compounds showed the molecular ion peak at *m/z* 582, a base peak **a** at *m/z* 446 ($M-C_{10}H_{16}$) and major fragments **b** and **c** at *m/z* 514 ($M-C_5H_8$) and *m/z* 391 ($M-C_{14}H_{23}$) in agreement with the presence of two terpenoid side chains on a cyclohexadienone ring (Fig. 1). The more detailed structure determination of these compounds was facilitated by extensive spectral studies, in particular UV and ^{1}H NMR (Table 1).

The good agreement of the UV spectra of methoxymethylesters **2** [λ_{max} nm (log ε): 250 (4.04), 290 (4.14), 365 (sh)] and **3** [λ_{max} nm (log ε): 256 (4.34), 292 (4.24), 360 (sh)] with that of inophylloidic acid **5** [λ_{max} nm (log ε): 245 (3.89), 313 (4.08), 355 (sh)] leaves no doubt that the new products contain an identical chromophore, while **4** possesses the same chromophore [λ_{max} nm (log ε): 235 (3.90), 288 (3.95), 354 (2.95)] as pseudoinophylloidic acid **6** [λ_{max} nm (log ε): 246 (3.96), 296 (4.04), 355 (3.24)], (11) favouring a very close relationship between these compounds, including structural features like the C_{10} and C_5 side chains and the dimethylchromanone ring [9, 10]. Although clearly related to inophylloidic acid (**5**), **2** and **3** are definitely distinct substances, since they can be easily separated on TLC plates. In their NMR spectra, the signals of the protons at position 22 and 23 and the corresponding coupling constants (Table 1) indicate the presence of a *trans*-2,3-dimethylchromanone ring, a frequently observed heterocycle moiety among Guttiferae constituents. The other chemical shift values of the ^{1}H NMR spectra are in excellent agreement with the published data of **5** [10, 11] (Table 1), and the assignments were further confirmed by a detailed analysis of the ^{1}H shift-correlated 2-D NMR spectra (COSY 45) [12] of **2** and **3**. In the COSY-spectrum, the methyl groups 10 and 11 of the dimethylallyl side chain could be easily identified due to the existence of a long-range coupling with H-8. Similarly, long-range coupling of the C-18 methyl protons with the *exo*-methylene protons at C-17 were observed. Especially, the rare occurrence of a cyclobutane derived from the equally unusual lavandulyl chain is readily recognised from the two remaining methyls singlets at high field (0.79 and 1.05 ppm) in the new compounds (Table 1).

The ^{13}C NMR spectra clearly establish the presence of a *n*-pentylchain instead of a *n*-propyl chain as in **5**. In addition they show the existence of a quaternary carbon atom (C-6, δ at about 60 ppm) on a cyclohexadienone ring substituted by two isoprene side chains as already described for compound **1** [5].

Small, but significant differences were observed in the ^{1}H NMR spectra, particularly in the chemical shifts (Table 1), of the signals for the two side chains. Much greater differences appear in their CD curves (see Experimental), suggesting that changes in the absolute configuration of one or several asymmetric carbon atoms in the vicinity of the chromophores may account for the observed variations of their spectral data. Since in ^{1}H NMR they are detected essentially in the signals attributed to the two isoprenoid chains, we propose that the absolute configuration at C-6 may differ in compounds **2** and **3**, although changes in the absolute configuration at C-22 and C-23 cannot be totally ruled out, since similar variations have been determined previously [11]. Unfortunately, the small amount available to us and the rather low stability of these compounds did not allow us to pursue this problem further. Structure **2a** and **3a** can therefore be attributed to the corresponding natural hydroxy-acids: caloverticillic acid A and caloverticillic acid B.

As previously described [9, 11], the numerous structural analogues of neoflavonoid acids show significant differences in their UV spectra depending on the position of the chromanone moiety and the chelated hydroxyl group on the cyclohexadienone ring.

Excepting the *n*-pentyl chain, **4** is related to pseudoinophylloidic acid **6** as revealed by a detailed analysis of its spectral data, particularly ^{1}H NMR (Table 1). While only small variations could be observed between the ^{13}C NMR spectra of **2** and **3**, much greater changes can be detected between spectra of compounds with different substitution patterns on the cyclohexadienone ring, for example the C-6 resonance of **2** and **3** is located at about δ 60 ppm while it appears at 57.5 ppm in the spectrum of **4**. The greatest difference is observed in the methoxy signals (64.8 ppm for **2** and **3**, 60.9 ppm for **4**), but the chemical shifts of the dimethylchromanone ring are also affected.

From the above data, formula **4** can be assigned to this compound. The corresponding natural acid, which we call

Table 1. ^{1}H NMR data of compounds **2**, **3**, **4**, **5** and **6**

H	2†	3†	4†	5‡	6‡
7	2.48 *dd* (14,6)	2.52 *dd* (13,9)	2.53 *dd* (14,8)		
	2.59 *dd* (14,6)	2.62 *dd* (13,9)	2.65 *dd* (14,9)		
8	4.67 *t* (6)	4.71 *t* (9)	4.75 *t* (9)	4.75 *t*	4.74 *t*
10	1.56* *s*	1.49* *s*	1.53 *s*	1.52 *s*	1.59 *s*
or 11	1.54 *s*	1.51* *s*	1.56 *s*	1.52 *s*	1.59 *s*
12	1.94 *dd* (13,3)	2.00 *dd* (13,3)	1.91 *dd* (13,3)		
12	2.20 *t* (13)	2.16 *t* (13)	2.10 *t* (13)		
13	1.42 *m*				
14		1.74 *m*	1.75 *m*		
15	2.41 *m*	2.36 *m*	2.35 *m*		
17	4.70 *br s*	4.77 *br s*	4.78 *br s*	4.46 *br s*	4.52 *br s*
17	4.45 *br s*	4.51 *br s*	4.54 *br s*	4.67 *br s*	4.78 *br s*
18	1.58* *s*	1.61* *s*	1.61* *s*	1.52 *s*	1.59 *s*
20	0.79 *s*	0.79 *s*	0.80 *s*	0.75 *s*	0.80 *s*
or 21	1.00 *s*	1.05 *s*	1.05 *s*	1.04 *s*	1.07 *s*
22	4.13 *dq* (10,6.5)	4.21 *dq* (10,6.5)	4.17 *dq* (12,6.5)	4.02	4.22
23	2.30 *m*	2.41 *m*	2.27 *dq* (12, 7)		
25	1.50 *d* (6.5)	1.49 *d* (6.5)	1.49 *d* (6.5)	1.48 *d*	1.57 *d*
26	1.17 *d* (7.5)	1.20 *d* (6.5)	1.16 *d* (7)	1.26 *d*	1.22
27	3.44 *m*	3.46 *m*	3.40 *m*	3.40 *m*	3.38 *m*
28	2.67 *m*	2.72 *m*	2.70 *m*		
28		2.67 *m*	2.67 *m*		
34	0.83 (*m*)	0.84 *m*	0.84 *m*		
Me(R_1)				0.90 *m*	0.90 *m*
OMe	3.85 *s*	3.88 *s*	3.75 *s*		
COOMe	3.56 *s*	3.58 *s*	3.57 *s*		

Chemical shifts are given in δ (ppm) relative to TMS. Coupling constants in Hz are given in parentheses.

*Confirmed by long-range coupling observed in the 2D-correlation spectrum.

†The spectrum was recorded at 500 MHz in $CDCl_3$.

‡The spectrum was recorded at 60 MHz [11].

verticillic acid C has the structure **4a** and is therefore the *n*-pentyl analogue of pseudoinophylloidic acid **6** [11]. It should be noted that the structure of these three new compounds fits current knowledge of the biosynthesis of the now well documented characteristic constituents of Guttiferae; differences of two methylene groups in the acid chains are in good agreement with their unsaturated fatty acid origin [13].

A series of molluscicidal tests was performed using several organs of *C. verticillatum* (leaves, seeds and bark) as well as with crude extracts of *C. inophyllum* (seeds) and *C. recedens* (bark). Their activity was compared with that of a crude mixture of caloverticillic acids and calofloride (**1**). All the extracts show 100% molluscicidal activity at 100 ppm while two of them (*C inophyllum* and *C. recedens*) are still active at 10 ppm, although less than **1**. Thus, the hitherto neglected Guttiferae species of Madagascar, which had never before been investigated for their potential use in the fight against schistosomiasis may constitute an interesting alternative to commercially available products, particularly since the above mentioned data compare favourably with those obtained from a large number of screening studies [14–17].

EXPERIMENTAL

MS were recorded on an AEI MS9 instrument (70 eV) in the electron impact mode and chemical ionization (NH_3). The NMR spectra were performed on a Bruker AM 500 spectrometer. The UV spectra were measured in EtOH and the IR spectra in $CHCl_3$. The CD was obtained in EtOH.

Plant material. The stem bark of *C. verticillatum* was collected in the forest of Fenerive (Eastern Madagascar) in 1984 and a voucher specimen of the plant material deposited at the Muséum d'Histoire Naturelle, Paris.

Extraction and isolation. The powdered bark (500 g) was extracted with CH_2Cl_2 in a Soxhlet. The crude extract (26 g) was separated on silicic acid (Mallinckrodt) by prep. HPLC using petrol–EtOAc to give two major fractions: 10.5 g (43%) of terpenoids and 8 g (31%) neoflavonoids contaminated by triterpenoids.

After chromatography over silica gel and elution with cyclohexane–EtOAc, the crystalline triterpenoids friedelin (15%) and α-amyrin (6%) were isolated and identified from their spectral data [7, 8]. 4 g of the second fraction were chromatographed over silica gel. Elution with cyclohexane–acetone 8:2 gave 180 mg of a crude mixture, which after further purification provided 35 mg of amorphous caloverticillic acids (0.13%).

Another sample (4 g) of the crude neoflavonoidic fraction was methylated with CH_2N_2 in MeOH for 48 hr. The mixture was heated with aq. HOAc and extracted with CH_2Cl_2. The residue was subjected to prep. HPLC (Miniprep Jobin-Yvon, 13 bars, 10 ml/mn, Silicagel: 0.010–0.040 mm, detection: UV). Elution with petrol–EtOAc gave three methoxy methyl esters **2** (20 mg, 0.5%), **3** (35 mg, 0.9%) and **4** (50 mg, 1.2%)

5-O-methyl caloverticillic acid A methyl ester **2**. Amorphous

$[\alpha]_D = -78.5°$, ($CHCl_3$, *c* 0.5). MS *m/z* (rel. int.): 582.3924 $[M]^+$ (2) (calc. for $C_{36}H_{54}O_6$582.3920), 567 (4), 514 (18) 499 (9), 483 (30), 446 (73), 432 (30), 431 (39), 391 (37), 377 (15), 375 (12), 359 (14), 357 (13), 69 (62), 55 (82), 41 (100). CD: (EtOH, *c* 0.12), $\Delta\varepsilon_{258}$ +8.25, $\Delta\varepsilon_{370}$-2.67. UV:λ_{max} nm (log ε): 250 (4.04), 290 (4.14), 365 (sh.). IR: ν_{max} cm^{-1}: 1725, 1675, 1620, 1590. ^{13}C NMR (125.76 MHz, $CDCl_3$ δppm); C: 196.7 (C-24), 192.7 (C-1), 185.5, 174.0 (C-29), 166.6, 145.8 (C-16), 134.3 (C-9), 113.6, 59.8 (C-6), 40.8 (C-19); CH: 118.3 (C-8), 77.4 (C-22), 48.5 (C-26), 48.4, 37.8, 31.0; CH_2: 108.7 (C-17), 41.6, 40.5, 37.8, 32.5, 31.8, 27.6, 26.1, 22.8; Me: 64.8 (Ar-OMe), 51.2 (OMe, ester), 25.6 (C-11), 24.7, 24.1, 23.7 (C-18, C-20, C-21), 19.4 (C-25), 17.9 (C-10), 14.1 (C-34), 12.2 (C-26).

5-O-*methyl caloverticillic acid B methyl ester* **3**. Amorphous $[\alpha]_D = 0°$, ($CHCl_3$, *c* 0.5). MS *m/z* (rel. int.): 582.3928 $[M]^+$ (2) (calc. for $C_{36}H_{54}O_6$582.3920), 514 (18), 500 (7), 499 (6), 483 (241), 446 (91), 432 (24), 431 (56), 391 (58), 377 (13), 375 (16), 359 (19), 357 (19), 69 (100), 55 (25), 41 (40). CD: (EtOH, c 0.2), $\Delta\varepsilon_{245}$-4.13, $\Delta\varepsilon_{280}$ +2.42, $\Delta\varepsilon_{322}$ +0.49, $\Delta\varepsilon_{365}$ −0.49. UV:λ_{max} nm (log ε): 256 (4.34), 292 (4.24), 360 (sh). IR: ν_{max} cm^{-1}: 1725, 1675, 1630, 1580. ^{13}C NMR (125.76 MHz, $CDCl_3$ δppm); C: 196.8 (C-24), 192.6 (C-1), 185.2, 174.0 (C-29), 166.7, 145.9 (C-16), 135.0 (C-9), 113,7, 60.0 (C-6), 40.6 (C-19); CH: 117.5 (C-8), 77.4 (C-22), 48.5 (C-23), 38.4, 30.9; CH_2: 108.6 (C-17), 41.8, 40.3, 37.8, 32.5, 31.9, 27.6, 26.2, 22.7; Me: 64.8 (Ar-OMe), 51.2 (OMe ester), 25.7 (C-11), 24.8, 24.2, 23.8 (C-18, C-20, C-21), 19.5 (C-25), 17.8 (C-10), 14.2 (C-34), 12.3 (C-26).

3-O-*methyl caloverticillic acid C methyl ester* **4**. Amorphous $[\alpha]_D = +6°$, ($CHCl_3$, *c* 1), MS *m/z* (rel. int.): 582.3924 $[M]^+$ (1) (calc. for $C_{36}H_{54}O_6$ 582.3920), 514 (12), 499 (5), 483 (7), 446 (46), 431 (22), 391 (58), 377 (6), 375 (17), 359 (35), 289 (28), 69 (100), 55 (36), 41 (57). CD: (EtOH; *c* 0.24), $\Delta\varepsilon_{247}$ −0.34, $\Delta\varepsilon_{257}$ +2.95, $\Delta\varepsilon_{316}$ +3.25. UV: λ_{max} nm (log ε): 235 (3.90), 288 (3.95), 354 (2.95). IR: ν_{max} cm^{-1}: 1735, 1675, 1630, 1595. ^{13}C NMR (125.76 MHz, $CDCl_3$ δ ppm); C: 196.2 (C-24), 189.0 (C-1), 188.5, 173.7 (C-29), 145.7 (C-16), 134.7 (C-9), 57.5 (C-6), 40.4 (C-19); CH: 117.7 (C-8), 80.3 (C-22), 48.7 (C-26), 46.0, 38.3, 31.6; CH_2: 108.8 (C-17), 40.7, 37.8, 33.2, 32.0, 29.7, 27.8, 26.8, 22.6; Me: 60.9 (Ar-OMe), 51.2 (OMe, ester), 25.7 (C-11), 24.7, 24.2, 23.8 (C-18, C-20, C-21), 19.0 (C-25), 18.1 (C-10), 14.1 (C-34), 10.9 (C-26).

Molluscicidal activity. The molluscicidal activity has been evaluated against young (3 weeks old) *Biomphalaria glabrata* for 24 hr at different concns (in ppm). The activity given corresponds to the rate of mortality (in %) [14].

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