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ANTIMICROBIAL DITERPENOID ALKALOIDS FROM *ERYTHROPHLEUM SUAVEOLENS* (GUILL. & PERR.) BRENAN

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ABSTRACT. An investigation of the stem bark of *Erythrophleum suaveolens* (Guill. & Perr.) Brenan yielded the known amide norcassaide (**1**) and a new diterpenoid alkaloid named norerythrosuaveolide (**2**) which was characterized as 7β-hydroxy-7-deoxo-6-oxonorcassaide. The structures were established on the basis of one and two-dimensional ^{1}H and ^{13}C NMR spectral data. The compounds showed potent antimicrobial activities against bacteria and yeasts.

KEY WORDS: *Erythrophleum suaveolens* (Guill. & Perr.) Brenan, Norcassaide, Diterpenoid alkaloid, Norerythrosuaveolide, Antimicrobial activities, Bacteria, Yeasts

INTRODUCTION

Erythrophleum suaveolens (syn: *Erythrophleum guineense*) is a large tree belonging to the Caesalpiniaceae family [1]. The bark decoction is used as an emetic, anti-inflammatory and analgesic. It is also used to dress wounds, to treat chickenpox and gangrenous sores, and as an ordeal poison [2]. The bark decoction of this plant is well known by traditional medicine practitioners in the Congo, the Democratic Republic of Congo (Zaïre) and, especially, by those in the Central and Eastern provinces of Cameroon who use it empirically for several ailments, including cardiovascular diseases and various inflammations. As part of a systematic search for anti-fungal and antibacterial agents from natural sources, the bio-guided fractionation of the stem bark extract of *E. suaveolens* furnished a chloroform extract which exhibited significant anti-fungal activity against 2 yeasts (*Candida albicans* and *C. krusei*) and antibacterial activity against 10 bacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella typhi*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Streptococcus pneumoniae*). Further purification of this fraction led to the isolation of two compounds: the known amide, norcassaide (**1**) and a new diterpenoid alkaloid, norerythrosuaveolide (**2**), which has been characterized as 7β-hydroxy-7-deoxo-6-oxonorcassaide. The present paper deals with the structure elucidation of these compounds as well as their antimicrobial activity.

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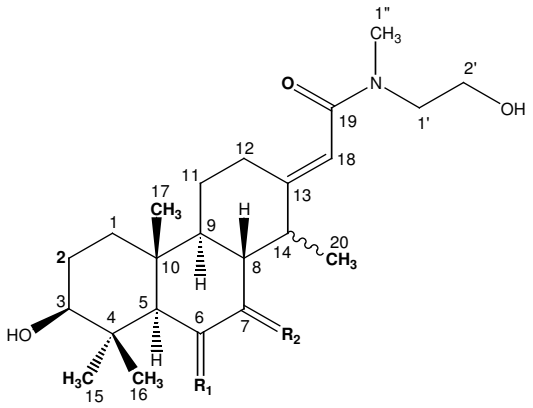


1 R_1 = H, H R_2 = O **2** R_1 = O R_2 = β-OH, H

EXPERIMENTAL

General

Melting points are uncorrected. ^{1}H NMR spectra were recorded at 400 and 500 MHz and ^{13}C NMR spectra at 100 and 125 MHz in methanol-d_4 using TMS as internal Standard. Sephadex was used for column chromatography (LH 20). Precoated silica gel plates (Merck, silica gel 60 F_{254}, 0.25 mm) were used for TLC.

Plant material

Erythrophleum suaveolens (Guill. & Perr.) Brenan was collected in the Yaoundé zone (Centre Province, Cameroon) in June 2000. A Voucher specimen N° 2644/SRFK has been deposited at the Cameroon National Herbarium.

Extraction, isolation and characterization

Air-dried and finely powdered stem bark of *E. suaveolens* (2 kg) was macerated in hexane (10 L) for 72 hours affording a hexane extract (15 g). The residual air-dried powder was treated with 10% aqueous NH_3 and macerated in $CHCl_3$ for 24 hours. After filtration and evaporation at reduced pressure, the resultant crude extract (90 g) was treated with 5% aq. HCl (3 L). The aqueous phase was made alkaline with aqueous NH_3 and extracted several times with $CHCl_3$. The $CHCl_3$ fraction was washed with water and dried ($MgSO_4$). Evaporation of the solvent furnished a fluffy extract (7 g). Half of this solid was chromatographed on Sephadex (LH 20) using $CHCl_3$-MeOH (1:1) and collecting 100 mL fractions. Identical fractions (TLC) obtained on elution with $CHCl_3$-MeOH (0.5:9.5) were combined and the solvent removed *in vacuo*. The resultant semi-solid was further purified by preparative chromatography using $CHCl_3$-MeOH (0.5:9.5) as elution solvent giving crystaline **1** (62 mg) and **2** (87 mg).

*Norcassaide (**1**).* $C_{23}H_{37}NO_4$, white crystals, m.p. 205-207 °C. U.V.: λ_{max} (nm, log ε) (254, 3.98). ESI-MS, positive mode (rel. int. %), *m/z* 392 $[M+H]^+$ (100), 414 $[M+Na]^+$ (13), 409 $[M+H_2O]^+$ (10). ^{1}H NMR and ^{13}C NMR (400 MHz and 100 MHz, methanol-d_4): Table 1.

*Norerythrosuaveolide (**2**)*. $C_{23}H_{37}NO_5$, solid white crystals, m.p. 204-205 °C. U.V.: λ_{max} (nm, log ε) (254, 3.98). ESI-MS, positive mode (rel. int. %), *m/z* 408 $[M+H]^+$ (100), 430 $[M+Na]^+$ (11), 815 $[2M+H]^+$ (35), 837 $[2M+Na]^+$ (12). ^1H and ^{13}C NMR (500 MHz and 125 MHz, methanol-d_4): Table 1.

Table 1. ^1H and ^{13}C-NMR spectral data* of compounds **1** and **2** recorded in methanol-d_4.

Carbon	**1**			**2**		
N°	δ_H	δ_C	DEPT	δ_H	δ_C	DEPT
1	1.87 m	38.0	CH_2	1.41 m 1.78 m	36.1	CH_2
2	1.68 m 1.89 m	25.2	CH_2	1.66 m 1.63 m	25.2	CH_2
3	3.23 m	79.6	CH	3,14 (dd, J = 5 and 11 Hz)	76.4	CH
4		37.2	C		36.2	C
5	1.30 m	52.9	CH	2.24 s	60.8	CH
6	2.36 m 2.41 m	39.7	CH_2		209.6	C
7		212.7	C	3.96 d (J = 11 Hz)	74.8	CH
8	2.28 dd (J = 4 and 13 Hz)	54.7	CH	1.67 m	49.0	CH
9	1.64 m	48.8	CH	1.73 m	44.0	CH
10		39.9	C		41.1	C
11	1.11 m	27.9	CH_2	1.84 m	24.5	CH_2
12	2.07 m 2.73 m	26.2	CH_2	2.09 m 2.76 m	23.3	CH_2
13		155.9	C		154.2	C
14	2.77 m	39.8	CH	2.77 dq (J = 13 and 7 Hz)	38.2	CH
15	0.95 s	28.1	CH_3	1.03 s	25.5	CH_3
16	1.02 s	13.7	CH_3	1.25 s	13.8	CH_3
17	0.84 s	15.3	CH_3	0.77 s	12.9	CH_3
18	5.89 s	116.5	CH	5.89 s	114.0	CH
	5.99 s	116.7		5.99 s	113.9	
19		171.3	C		168.8	C
20	1.04 d (J = 7 Hz)	15.6	CH_3	1.19 d (J = 7 Hz)	11.2	CH_3
1'	3.49 m	53.7	CH_2	3.48 m	51.2	CH_2
	3.51 m	50.8		3.50 m	48.3	
2'	3.65 t (J = 6 Hz)	60.6	CH_2	3.67 t (J = 6 Hz)	58.1	CH_2
	3.69 t (J = 6 Hz)	60.2		3.70 t (J = 6 Hz)	57.7	
1"	2.97 s	33.5	CH_3	2.97 s	31.0	CH_3
	3.10 s	38.1		3.10 s	35.5	

* Assignments based on COSY, HMQC and HMBC.

Antimicrobial assay

The antimicrobial activity of compounds **1** and **2** was studied using 12 microbial cultures belonging to 9 aerobic bacterial species (*Escherichia coli* LMP0101U, *Staphylococcus aureus* LMP0206U, *Proteus vulgaris* LMP0103, *Klebsiella pneumoniae* LMP0210U, *Salmonella typhimurium* LMP0413, *Pseudomonas aeruginosa* LMP0102U, *Streptococcus faecalis* (LMP0207U), *Salmonella typhi* LMP0209U and *Streptococcus pneumoniae* LMP0210U), one anaerobic bacterium (positive beta-lactamase, *Neisseria gonorrhoeae* LMP0412) and 2 *Candida* species (*C. albicans* LMP0204U and *C. krusei* LMP0311U). These strains were clinically isolated from the urogenital discharges of patients in the *Centre Pasteur du Cameroun* health institution and monitored in the Laboratory of the Applied Microbiology and Molecular Pharmacology (LMP) of the University of Yaoundé I. The strains were activated at 37 °C for 24 hours on nutrient agar (aerobic bacteria), Sabouraud glucose agar (fungi), or 48 hours on the Mueller Hinton agar supplemented with 1% polyvitex and 5% defribrinated sheep blood (MHAPB) in 10% CO_2 atmosphere for *Neisseria gonorrhoeae*. The antimicrobial activities

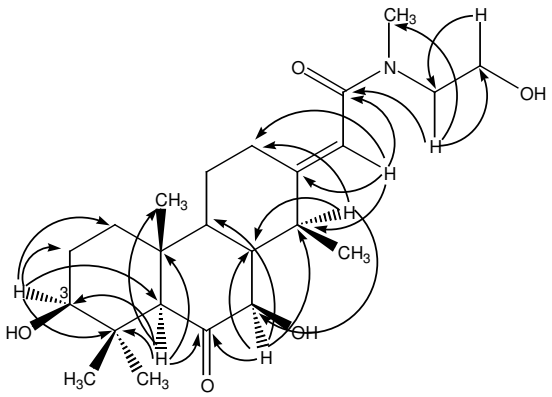
(Table 2) were evaluated on the basis of the minimal inhibition concentration (MIC) by Agar hole diffusion method [3] in series for *Neisseria gonorrhoeae*, and the macrodilution method [4] for other microorganisms. As the molecules were insoluble in water, several other solvents were tested. It was found that phosphate buffer pH 4.4 and 4.7 gave optimal results respectively for compounds **2** and **1**. These solvents were consequently used in testing. Gentamicin (Bacteria) and Nystatin (yeasts), diluted in water, were used as reference antibiotics.

RESULTS AND DISCUSSIONS

The crude chloroform extract, obtained from the stem bark of *Erythrophleum suaveolens* as described above was purified by chromatography to furnish the known amide norcassaide (**1**) and a closely related diterpenoid alkaloid **2**.

Compound **2** was isolated as colourless crystals from chloroform (m.p. 204-205 °C) and gave positive Draggendorf's and Mayer's tests, suggesting the presence of nitrogen. The UV spectrum, λ_{max} (nm, log ε): (254, 3.98) and (366, 4.14), was compatible with the presence of a substituted α,β-unsaturated carbonyl group, while the IR spectrum showed bands due to hydroxyl (3480 cm^{-1}), amide carbonyl (1593 cm^{-1}), conjugated C=C (1667 cm^{-1}) and amide C-N (1186 cm^{-1}) functions. The ESI mass spectrum of **2** exhibited an $[M+H]^+$ peak at *m/z* 408, indicating the molecular formula $C_{23}H_{37}NO_5$ with six unsaturations and confirming the presence of nitrogen. Thus compound **2** contained an additional hydroxyl group relative to **1** ($[M+H]^+$ peak at *m/z* 392). The ^1H and ^{13}C NMR spectroscopic data of **2** (Table 1) were assigned using ^1H-^1H COSY, HMQC and HMBC techniques. The ^{13}C NMR spectrum exhibited 23 carbon atoms, including two carbonyls, one an amide (δ_C 168.8) and the other a ketone (δ_C 209.6), and two sp^2 carbons of a trisubstituted double bond (δ_C 114.0 and 154.2). A DEPT experiment revealed 18 carbon atoms attached to a total of 34 hydrogen atoms: 5 methyl, 6 methylene and 7 methine groups. Three of the carbons, a methylene (δ_C 57.7) and two methines (δ_C 74.8 and δ_C 76.4), were oxygenated. The remaining three degrees of unsaturation suggested that **2** had a tricyclic diterpenoid skeleton as in **1**.

The ^1H NMR spectrum of **2** (Table 1) showed a close similarity to that of **1** (Table 1) except for the presence of a singlet at δ_H 2.24 (H-5) and a carbinol proton H-7 (δ_H 3.96, d, J = 11 Hz) associated with a hydroxylated carbon C-7 (δ_C 74.8). It was apparent that **2** had the same structure as **1** apart from the substitution of ring B. H-7 showed a diaxial coupling with H-8. Thus the hydroxyl group is beta [5-7]. As erythrosuavine in our earlier investigation on *E. suaveolens* [7], the 7β-OH orientation could explain the observed deshielding effect on the C-20 methyl protons which appeared in **2** as a doublet (J = 7 Hz) at δ_H 1.19 relative to **1** (δ_H 1.04, J = 7 Hz). This observation confirmed the β-orientation of the C-20 methyl group located on C-14 in compound **2** instead of the α-orientation as in **1**. The ketonic carbonyl group must be situated at C-6. HMBC correlations from H-5 (Scheme 1) to the carbons at δ_C 209.6 (C-6), 36.2 (C-4), 74.8 (C-7), 44.0 (C-9) and 76.4 (C-3) confirmed the substitution pattern of ring B. The expected *E* configuration of the $\Delta^{13,18}$ double bond [5, 7-9] is evident from the deshielding effect of the side chain carbonyl function (δ_C 168.8) of one of the two allylic protons (δ_H 2.09 and 2.76) at C-12 (δ_C 23.3). The two methylene proton signals at δ_H 3.50 (t, J = 6 Hz) and 3.70 (t, J = 6 Hz) were clearly visible on the ^1H-^1H COSY spectrum as an isolated correlation system It is noteworthy that the signals of H-18, N-methyl, N-methylene and O-methylene groups were doubled, as expected, in both the ^1H and ^{13}C NMR spectra of **1** and **2** as a result of restricted rotation about the amide bond [10]. Hence, compound **1** is the known compound norcassaide [10, 11] while **2** is 7β-hydroxy-7-deoxo-6-oxonorcassaide, named norerythrosuaveolide, which to our knowledge has not previously been described in the literature.



Scheme 1. Some HMBC correlations of compound **2**.

Compound **1** and **2** showed strong antimicrobial activities with a large spectrum of activity (Table 2). The Minimum Inhibition Concentration (MIC) of compound **1** varied from 9.76 µg/mL (*C. krusei*) to 39.06 µg/mL (*C. albicans*) on yeasts and from 39.06 µg/mL (*K. pneumoniae* and *N. gonorrhoeae*) to 156.00 µg/mL (*E. coli*, *P. aeruginosa*, *S. typhimurium* and *S. aureus*) on bacteria. Those of compound **2** varied from 9.76 µg/mL (*C. krusei*) to 19.50 µg/mL (*C. albicans*) on yeasts and from 9.76 µg/mL (*N. gonorrhoeae*) to 312.50 µg/mL (*S. typhimurium* and *S. aureus*) on bacteria. It appears that the activity of compound **2** observed on *N. gonorrhoeae* is greater than those of Gentamicin and Nystatin for *C. albicans* and *C. krusei*. Compound **1** also exhibited a better antimicrobial activity on *C. krusei* than Nystatin. *C. krusei* appears to be the most sensitive fungi to compound **1** and **2** whereas *N. gonorrhoeae* is the most sensitive bacterial strain.

Table 2. Minimal inhibition concentration* (MIC) of compound **1** and **2** and the reference antibiotics.

Tested microrganisms	MIC (µg/mL) of the compounds		
	Norcassaïde (**1**)	Norerythrosuaveolide (**2**)	GM/N**
Bacteria			
Escherichia coli	156	39	10
Klebsiella pneumoniae	39.06	39.06	10
Neisseria gonorrhoeae	39.06	**9.76**	20
Pseudomonas aeruginosa	156	156	10
Proteus vulgaris	78.12	78.12	5
Salmonella typhi	78.12	78.12	5
Salmonella typhimurium	156	312.5	10
Staphylococcus aureus	156	312.5	10
Streptococcus faecalis	78.12	78.12	10
Streptococcus pneumoniae	78.12	78.12	20
Yeasts			
Candida albicans	39.06	**19.5**	30
Candida krusei	**9.76**	**9.76**	30

*Results of the MIC recorded as mean of triplicated experiments. **GM: gentamicin for bacteria N: nystatin for yeasts.

ACKNOWLEDGMENTS

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