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Isolation, Characterization, and Biological Activities of Gossweilerine, an Unusual Quaternary Alkaloid from *Drypetes Gossweileri*

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A B S T R A C T

A new quaternary alkaloid, gossweilerine, together with four known compounds was isolated from the barks of *Drypetes gossweileri*. The structures of the new and known compounds were established on the basis of one- and two-dimensional NMR and HR-EI-MS data as gossweilerine (**1**), gossweilone (**2**), friedelin, 3,7-dioxofriedelan, and 3-oxo-16β-hydroxypachysonol. Gossweilerine (**1**) and gossweilone (**2**) displayed a cytotoxic activity on brine shrimp (*Artemia saline*), with a LD_{50} 7.12 and 19.31 µg/mL, respectively and *in-vitro* phytotoxicity with growth regulation of 14.6 and 54.9% respectively at concentration of 10µg/mL. These compounds were also evaluated for antibacterial and antifungal activities. Compound **1** exhibits significant activities against *E. coli*, *S. aureus*, *S. typhii*, *T. longiformis*, and *M. canis* while compound **2** exhibits significant activities against *E. coli* and *S. typhii*, and weak to moderate inhibitory activities against all fungi.

Keywords: *Drypetes gossweileri;* Euphorbiaceae; Alkaloid; Antimicrobial; cytotoxicity; Phytotoxicity

A R T I C L E I N F O

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1. Introduction

Plants belonging to the Drypetes genus (Euphorbiaceae) are widely used in traditional medicine in West and Central Africa for the treatment of diverse infections such as sinusitis, swelling, boils, gonorrhea, and dysentery [1–4]. *Drypetes gossweileri* is a tree (length 40 m, width 1 m, approx.) used in dermatitis, ocular, and respiratory manifestations [5]. A variety of compounds have been isolated from Drypetesgenus including triterpenoids [6, 7], sterols [7], sesquiterpene lactones [8], lignans [8], diterpenes [9], xanthones [9], anthraquinones [10].

During the course of chemical study on Cameroon medicinal plant *D. gossweileri* and the search for potential anticancer agents from Cameroon medicinal plants, we isolated five compounds, including a new quaternary alkaloid named gossweilerine (**1**), the known podocarpane derivative, gossweilone (**2**), and three known triterpenoids. In this paper, we report the structure elucidation for the new isolated compound **1**, the growth inhibitory effects against strains of *Saccharomyces cerevisiae*, the antibacterial, antifungal, cytotoxic, and phytotoxic activities of compounds **1–2**.

2. Materials and Methods

General experimental procedures: UV spectra were recorded on a Bio-TeK spectrophotometer. Melting points were determined on a Büchi 535 melting point apparatus and are uncorrected. IR spectra were recorded on a JASCO 302-A spectrophotometer in $CHCl_3$. The EI-MS (70 eV) were measured on a Varian AAT 311A mass spectrometer, and HR-EI-MS were taken on a JEOL HX110 mass spectrometer. 1D and 2D NMR spectra were run on Bruker AMX 300 MHz NMR spectrometer. The chemical shifts are given in ppm (δ), relative to TMS as internal standard, and coupling constants are in Hz. Column chromatography was carried out on silica gel (70 – 230 mesh, Merck). TLC was performed on Merck precoated aluminum silica gel 60 F254 sheets. The plates were checked under UV light (254 and 366 nm) and developed with vanillin and H_2SO_4 in EtOH. A molecular device spectrophotometer was used for measurement of enzyme inhibition.

Plant material

Stem barks of *Drypetes gossweileri* (DG, Fam. Euphorbiaceae) were harvested in Mbalmayo, Center Province of Cameroon in May 2008 and the voucher specimen (N°5749/HNC) was deposited at the National Herbarium of Yaounde, Cameroon.

Extraction and Isolation

The air-dried barks (10 kg) of *D. gossweileri* were extracted at room temperature with MeOH (4L x 3) for 72 h. A preliminary biological screening of this extract exhibited medium to good antibacterial, antifungal, phytotoxic, and cytotoxic activities. The methanol extract (430 g) was evaporated to dryness, defatted with hexane, suspended in H_2O and extracted with ethyl acetate. The hexane (26 g), and ethyl acetate (9 g) extracts were subjected to the above tests. The EtOAc extract, which showed appreciable activity, was subjected to flash CC on silica gel by gradient elution with n-hexane, n-hexane-EtOAc, EtOAc, EtOAc – MeOH and finally MeOH. Five fractions (Fr.1 – Fr.5) were generated in this manner; gossweilerine (**1**) (75 mg) was obtained directly from Fr.5 (Hex – EtOAc 40/60) by recrystallization with EtOAc. Fr. 4 (Hex – EtOAc 60/40) was re-chromatographed by CC using Hex – EtOAc 60/40, followed by prep. TLC (in Me_2CO) from which gossweilone (**2**) (128 mg, Rf 0.34) was obtained. Fractions Fr.1, Fr.2, and Fr.3 were combined (2.07 g) after TLC [Hex-EtOAc (70:30)] indicated that they contained similar compounds. The combined fractions were further rechromatographed by CC using Hex-EtOAc (70:30) to yield friedeline (8 mg), 3,7-dioxofriedelane (16 mg), and 3-oxo-16β-hydroxypachysonol (12 mg).

***In-vitro* antibacterial bioassay**

The microbial cultures were originally obtained from American Type Culture Collection (ATCC). In *vitro* antibacterial activity was screened against six bacterial strains: *Bacillus subtilis* (ATCC-11774), *Escherichia coli* (ATCC-25922), *Staphylococcus aureus* (ATCC-25923), Shigella flexneri, *Pseudomonas aeruginosa*, and *Salmonella typhi* using agar-well diffusion method. Two to eight hours old bacterial inoculums containing approximately 10^4–10^6 colony forming units (CFU)/mL were used in these assays.

The wells were dug in the media with the help of a sterile metallic borer with centers at least 24 mm. Recommended concentration (100 μL) of the test sample (1 mg/mL in DMSO) was introduced in the respective wells. Other wells supplemented with DMSO and reference antibacterial drug, Cefixime served as negative and positive controls, respectively. The plates were incubated immediately at 37 °C for 24 hours. Activity was determined by measuring the diameter of zones showing complete inhibition (mm). In order to clarify any participating role of DMSO in the biological screening, separate studies were carried out with the solutions alone of DMSO and they showed no activity against any bacterial strains.

Antifungal activities

Extracts and compounds were tested against various pathogens, namely, *Trichophyton,* Longifusus ATCC 22397, *Candida albicans* ATCC 2192, *Aspergillus flavus* ATCC 1030, *Microsporum canis* ATCC 9865, *Fusarium solani* ATCC 11712 and *Candida* glabrata by using tube diffusion test. The Miconazole (200µg/mL), and Amphotericin-B (200 µg/mL) were used as standards drugs. Stock solutions of pure compounds (12µg/mL) were prepared in sterile DMSO. Sabouraud dextrose agar was prepared by mixing Sabouraud (32.5 g), glucose agar (4%), and agar-agar (4 g) in 500 mL of distilled water followed by steamed dissolution, 4 mL of the media being dispensed into screw capped tubes and autoclaved at 121°C for 15 min. Test compounds were added (66.6 µg/mL) from the stock solution to non-solidified Sabouraud agar medium (50°C). Tubes were allowed to solidify at room temperature and inoculated with 4 mm diameter portion of inoculums derived from a 7 days old respective fungal culture. For non-mycelial growth, an agar surface streak was employed.

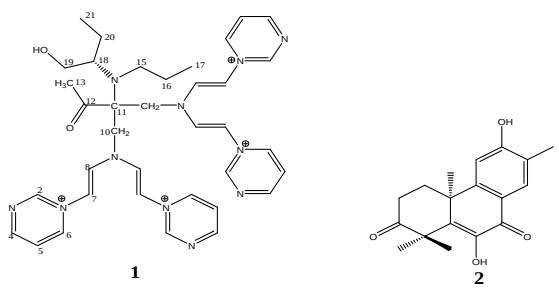
The tubes were incubated at 27–29°C for 7–10 days and the growth in the compound containing medium was determined by measuring the linear growth (mm) and growth inhibition with respective control. The amount of growth inhibition was calculated as Inhibition (%) = 100(*A* –*B*)/*A*, where *A* = diameter of fungal colony in control plate, B = Diameter of fungal colony in test plate.

Brine shrimp lethality assay

Sea water made by 38 gm (3.8%) sea salt was dissolved in one liter of distilled water and then filtered off. This Sea water was taken in a small tank and shrimp eggs were added to one side of the divided tank and this side was covered. The brine shrimp specie Artemia salina Leach eggs were allowed for two days to hatch and mature as nauplii (second instar larvae). The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforations in the dam. These nauplii were taken for bioassay. Then 10, 100, and 1000 µg of the test materials were taken in each vial and 5 mL of sea water added to each vial containing 10 brine shrimp nauplii. Three vials were used for each concentration. Control wells received 100 µL of the corresponding solvent (DMSO) used for the tested samples, instead of extract, and had the same final volume (5 mL) as experimental wells. A magnifying glass was used for convenience of counting of the nauplii. After 24 hours, the vials were observed and the number of survivors in each vial were counted and noted. From this data, the percentage of mortality of nauplii was calculated at each concentration.

Phytotoxic (Lemna) bioassay

Lemna minor was prepared by aseptic technique. Inorganic E medium (about 80 mL per compound) was prepared [KH_2PO_4 680.0, KNO_3 1515.0, $Ca(NO_3).24H_2O$ 1180.0, $MgSO_4.7H_2O$ 492.0, H_3BO_3 286.0, $MnCl_2.4H_2O$ 3.62, $FeCl_3.6H_2O$ 5.40, $ZnSO_4.7H_2O$ 0.22, $CuSO_4.5H_2O$ 0.22, $Na_2MoO_4.2H_2O$ 0.12 and EDTA 11.2 mg/mL]; KOH pellets was added to make the pH 5.5–6.0. 10 vials per dose were prepared for testing. Then 15 mg of sample was dissolved in 15 mL of solvent. 1000, 100, and 10 mL solution were added to vials and allowed the solvent to evaporate overnight. 2 mL of E medium was then added and a single plant containing a rosette of three fronds to each vial. The vials were placed in glass dish filled with about 2cm water, seal container with stopcock grease and glass plate. These dishes were placed with vials in growth chamber for seven days in growth chamber with temperature range of 27 to 29°C and 24 hours of fluorescent and incandescent lights. On days 3 and 7, the number of fronds per vial was counted, recorded, and analyzed.



1 **2**

Figure 1: Structures of compounds **1** and **2**

Gossweilerine (1)

White amorphous powder; $[\alpha]_D^{20}$ +11.7°(c 0.1, MeOH); UV (MeOH) λ_{max}(logε) 217 (4.31), 283 (4.22), 306 (4.02) nm; CD ($0.84.10^{-3}$ M, MeOH) λ_{max} ($\Delta\varepsilon$) (nm): 239 (+0.51), 278 (-1.13); IR $\neg^{KBr}_{max}$ cm^{-1}: 3450 – 3400 (OH), 3050, 1731 (C=O), 1603 (C=C), 1512, 1470, 1220, 940; ^{1}H- and ^{13}C-NMR ($CDCl_3$), **(**Table 1**)**; HR-EI-MS *m/z* 665.1651 $[M]^+$ (calculated for $C_{30}H_{34}O_7$, 665.1639).

3. Results and Discussion

The stem barks of Drypetes gossweileri were powdered and extracted with MeOH, and the solvent was evaporated under vacuum. The methanol extract was defatted with hexane, suspended in H_2O and extract with EtOAc. The EtOAc extract was chromatographed on a column of silica gel, eluted with n-hexane, EtOAc, and MeOH in increasing polarities to afford friedelin [11], 3,7-dioxofriedelan [11], 3-oxo-16β-hydroxypachysonol [12], gossweilone (2) [13], and a new alkaloid, gossweilerine (**1**). Gossweilerine (**1**) was obtained as a white solid, positive to Dragendorff reagent.

The molecular formula was determined as $C_{36}H_{47}N_{11}O_2$ from the HR-EI-MS (m/z 665.1651). Its IR spectrum showed characteristic absorption bands at 3433 (free OH or NH_2), 1731 (carbonyl), and 1603 cm^{-1} (C=C). The ^{13}C-NMR and DEPT spectrum of **1** showed signals of one carbonyl group (δ_C 203.1), four aromatic CH carbons (δ_C 160.3, 154.1, 151.7, and 139.7), two olefinic CH carbons (132.8 and 115.6), one deshielded quaternary carbon (δ_C 74.7), one deshielded methine (δ_C 59.7), five methylenes including three bearing an oxygen or a nitrogen atom, and three methyles groups (δ_C 11.8, 12.8, and 23.4). The chemical shifts and couplings of the four aromatic protons at δ_H 9.77 (s), 9.89 (dd, J=5.6, 1.6 Hz), 9.03 (dd, J=6.8, 6.8 Hz), and 9.41 (dd, J=6.8, 1.6 Hz) were indicative of a pyrimidine nucleus. Two doublets were also observed at δ_H 6.51 (d, J=16.3Hz) &7.39 (d, J=16.3 Hz) corresponding to two olefinic protons. The chemical shifts of these two protons were in agreement with a linkage to a heteroatom. This fragment should probably correspond to N-CH=CH-N moiety.

The HMBC cross-peaks of H-7 with C-2 (δ_C 154.1) and C-6 (δ_C 151.7) suggested a connection among C-2, C-3, and C-7 (δ_C115.6) through a nitrogen atom, and hence, were indicative of a N-substituted pyrimidine nucleus. The other olefinic proton at δ_H 7.39 (d, J=16.3 Hz) showed a HMBC correlation with the methylene at δ_C 61.2. The geometry of the double bond was determined to be E, from the observed coupling constant of the olefinic proton which is 16.3 Hz. The NOESY spectrum fully supported this observation. The most striking feature of the ^{1}H-NMR spectrum was the intensities of the different protons. The signals of the pyrimidine nucleus and the olefinic protons integrated for four protons each, suggesting the presence of four pyrimidine nucleus and four carbon-carbon double bonds. Furthermore, the methylene protons at δ_H 4.61 were clearly twice as intense as other methylene protons. The ^{1}H- and

^{13}C-NMR spectral features of **1**, together with its molecular formula of $C_{36}H_{47}N_{11}O_2$, suggested a symmetrical structure. Detailed analysis of the ^{1}H–^{1}H COSY, HMBC, and NOESY spectra of **1** allowed construction of four partial structures **1a, 1b, 1c, and 1d** (Figure 1).

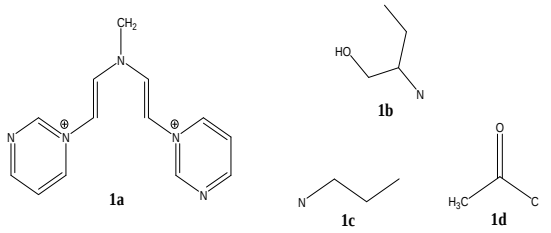


Figure 2: Partial structures observed in compound **1**

The COSY experiment showed a correlation series beginning with the methine at δ_C/δ_H 59.7/1.41 assigned to H-18, which was coupled with the oxymethylene at δ_C/δ_H 60.5/3.76 assigned to CH_2-19, and with the CH_2-20 methylene signal at δ_H 1.56. These latter protons were correlated to the H-21 protons at δ_H 1.05 attributed to the methyl protons. The presence of a hydroxyl group at the C-19 was evidenced by the HMBC experiment which showed the correlations of the H_3-19 proton with the carbons C-18 and C-20. Based on the above discussion, the partial structure **1b** was elucidated as 2-aminobutan-1-ol derivative. The partial structure **1c** was elucidated by analysing the ^{1}H–^{1}H COSY spectrum. In the ^{1}H–^{1}H COSY spectrum, the signal at δ_H 2.86 (br t, J=6.8 Hz, CH_2-15) showed cross-peaks with H_2-16 at δ_H 1.51 (2H, m), which in turn showed cross-peak with the methyl protons H_3-17 at δ_H 0.99 (t, J = 7.1 Hz). Hence the partial structure **1c** was deduced from the above data. Structural assessment of **1a** was effected by analysis of ^{1}H- and ^{13}C-NMR spectroscopic data. Allocation of signals was facilitated by COSY, DEPT, NOESY, HMQC, and HMBC experiments. The ^{13}C-NMR spectrum showed a carbonyl signals at δ_C 203.1, corresponding to the ketone C-12. The resonance of the quaternary C-11 was significantly deshielded at δ_C 74.7 indicating N-substitution. In the HMBC spectrum, a three-bond correlation is observed from the methyl protons H_3-13 to the C-11 leading to the partial structure **1d**. The HMBC data summarized in Figure 2 allowed us to connect the both partial structures.

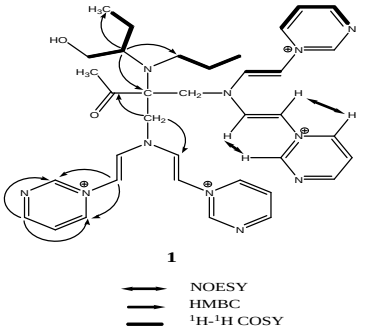


Figure 3: Selected ^{1}H-^{1}H COSY, HMBC, and NOESY for gossweilerine (**1**).

Long range correlations observed in the HMBC spectrum from methine proton at δ_H 1.41 (H-18) to the carbons at δ_C 74.7 (C-11) and 51.8 (C-15) as well as from methylene protons at δ_H 2.86 (H-15) to the carbons at δ_C 74.7 (C-11) and 59.7 (C-18), and from δ_H 4.61 (H-10) to the carbons at δ_C 74.7 (C-11), 59.7 (C-18), and 51.8 (C-15) established the proposed arrangement. Further analysis of the 2D NMR spectrum also allowed the complete assignment of its ^{13}C-NMR spectroscopic data (Table 1). The presence of asymmetric carbon (C-18) in **1** justified its optical rotation $[\alpha]_D^{20}$=+11.7°. The absolute configuration of C-18 was determined to be in S form, since the HPLC analysis of **1** with CD detection gave a negative Cotton effect at 278 nm ($\Delta\varepsilon$ −1.13) and a positive Cotton effect at 239 nm ($\Delta\varepsilon$ +0.51)[14]. Furthermore, the optical rotation value of **1** is similar to that of (S)-(+)-2-aminobutan-1-ol $[\alpha]_D^{20}$+10°, purity≥98%, Sigma-Aldrich), indicating that the two compounds have the same configuration. Thus, gossweilerine (**1**) was determined as 4-(bis ((E)-2-(pyrimidium-1-yl) vinyl) amino)-3(S)-((bis ((E)-2-(pyrimidium-1-yl) vinyl) amino)-methyl)-3-((1-hydroxy butan-2-yl) (propyl) amino) butan-2-one, an unprecedented quaternary alkaloid with four pyrimidine nucleus.

Table 1: ^{1}H and ^{13}C NMR data for compound **1** (δ in ppm, J in Hz, $CDCl_3$)

Position	δ_C	δ
2	154.1	9.77, s
4	160.3	9. 89, dd (5.6, 1.6)
5	139.7	9.03, dd (6.8, 6.8)
6	151.7	9.41, dd (6.8, 1.6)
7	115.6	6.51, d (16.3)
8	132.8	7.39, d (16.3)
10	61.2	4.61, s
11	74.7	-
12	203.1	-
13	23.4	2.29, s
15	51.8	2.86, br t (6.8)
16	22.4	1.51, m
17	12.8	0.99, t (7.1)
18	59.7	1.41, m
19	60.5	3.76, d (7.4)
20	21.1	1.56, br t (6.8)
21	11.8	1.05, t (7.8)

EtOAc extract and compounds **1** and **2** were evaluated for their growth inhibitory effects toward Artemia salina of Saccharomyces cerevisiae using etoposide as positive control. As shown in Table 2, compounds **1** and **2** exhibited cytotoxicity with LD_{50} 7.12 and 19.31µg/mL, respectively. The extract was more active than Compound **1** (LD_{50}13.69 µg/mL), but less active than compound **2**.

Table 2: Brine shrimp (*Artemia saline*) lethality bioassay of compounds and extract of *Drypetes gossweileri*

Samples	LD_{50} (µg/mL)	Probability (G value)
Extract	13.69	0.21
1	7.12	0.29
2	19.31	0.11
Etoposide	7.60	-

Extract and compounds **1** and **2** were screened for their antibacterial and antifungal activities against four Gram–negative bacteria (*Escherichia coli*, Shigella flexneri, *Pseudomonas aeruginosa, and Salmonella typhi*), two Gram–positive bacteria (*Bacillus subtilis and Staphylococcus aureus*) and six fungal strains (*Trichophyton longifusus, Candida albicans, Aspergillus flavus,* Microsporum canis, *Fusarium solani,* and *Candida glabrata*). Imipenem was used as standard antibacterial, and miconazole and amphotericin B were used as antifungal reference compounds. The zone of inhibition and percentage inhibition were determined using agar-well diffusion method and tube diffusion test, respectively. In antifungal activities, compound **1** showed significant inhibitory activity against *T. longiformis* and *M. canis* with inhibitions of 91% and 93% respectively, while compound **2** showed weak to moderate inhibitory activities against all fungi. The extract showed weak activity against *T. longiformis, A. flavus*, *M. canis*, and *F. solani*. The results of antibacterial activities of extract and compounds **1** and **2** are shown in Table 4. Compound **1** exhibits significant activities against *E. coli*, *S. aureus*, and *S. typhii* while compound **2** exhibits significant activities against *E. coli* and *S. typhii*. The extract showed weak activity against *E. coli*, *S. aureus*, and *S. typhii*. Only compound **2** showed marginal antibacterial activity against *P. aeruginosa*.

In vitro phytotoxicity (Lemna) bioassay of compounds **1** and **2** and extract of *Drypetes gossweileri* was also evaluated. As shown in Table 5, compounds **1** and **2** exhibited 54.9% and 23.1% growth regulation at concentration of 10 µg/mL, respectively, however lower than that of paraquat, the reference compound for this assay. The extract was less active than the isolated compounds.

Table 3: *In-vitro* antifungal activities of compounds and extract of *Drypetes gossweileri*

Samples	Percentage inhibition					
	T. longiformis	***C. albican***	***A. flavus***	***M. canis***	***F. solani***	***C. glutamate***
Extract	50.0	-	12.18	67.4	9.52	-
1	91	-	27	93	18	6
2	37	13	21	49	41	17
Miconazole	70	110.8	N/A	98.4	73.25	110.8
Amphotericin B	N/A	N/A	20.10	N/A	N/A	N/A

Values are % inhibition of radial growth. Concentration of samples = 400 µg/mL of DMSO for extract and 200 µg/mL for pure compounds. Incubation temperature (period) = 28 ± 1 °C (7 days). (–) = No activity. N/A not applicable.

Table 4: *In-vitro* antibacterial activities of compounds and extract of *Drypetes gossweileri*[a]

Samples	Zone of inhibition (mm)					
	E. coli	***B. subtilis***	***S. flexenari***	***S. aureus***	***P. aeruginosa***	***S. typhii***
Extract	13	-	-	16	-	14
1	31	-	-	28	12	32
2	27	-	-	13	-	31
Imipenem	33	30	35	43	25	40

[a]The values indicate zone of inhibition of microbial growth (mm). Samples were tested at 3 mg/mL in DMSO for crude extract and 1 mg/mL for pure compounds. [- (<9) = no activity; 9-12 mm = non-significant; 13-15 = weak; 16-18 good; > 18 = significant].

Table 5: *In -vitro* phytotoxicity (Lemna) bioassay of compounds and extract of *Drypetesgossweileri*

Samples	Growth regulation (%) in different conc. (µg/mL)		
	1000	100	10
Extract	100.0	60.6	14.6
1	100	79.1	54.9
2	100	48.6	23.1
Paraquat	100		

Test samples conc. 0.902 µg/mL

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