Novel antibacterial triterpenoid from *Combretum padoides* [Combretaceae]

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Dedicated to Professor Berhanu M. Abegaz on the occasion of his 60th birthday

Abstract
The dichloromethane extract of *C. padoides* leaves was subjected to antibacterial activity guided fractionation against *Staphylococcus aureus* to afford a new oleanene-type triterpenoid glycoside identified as 1α,23β-dihydroxy-12-oleanen-29-oic-acid-23β-O-α-4-acetylrhamnopyranoside (**1**) together with two known compounds 1,22-dihydroxy-12-oleanen-30-oic acid (**2**) and 24-ethylcholesta-7,22,25-trien-O-β-D-glucopyranoside (**3**) on the basis of 1D NMR, 2D NMR and ESI-MS analysis. Compounds **1** and **2** had a reasonable antibacterial activity [MIC of 0.031 and 0.063 mg/ml] against *S. aureus* and *Escherichia coli*. All compounds were non-cytotoxic. The results indicate that pentacyclic triterpenes from *C. padoides* have some antibacterial activity with no cytotoxic activity.

Keywords: *Combretum padoides*, Combretaceae, triterpenoid, antibacterial, cytoxicity

Introduction

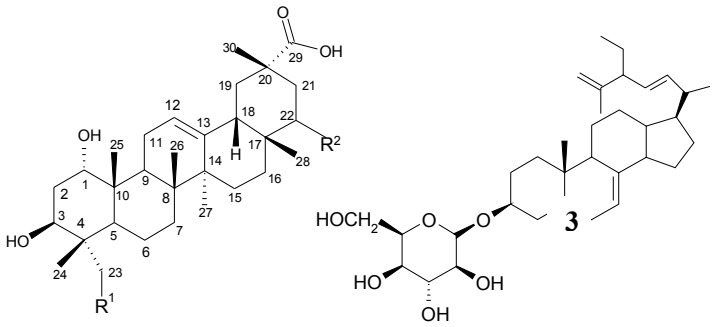
In our preliminary screening of members of the Hypocrateroptis section (*C. imberbe* Wawra, *C. padoides* Engl. & Diels, *C. celestroides* Welw. ex M. A. Lawson ssp *celestroides* and *C. celestroides* Welw. ex M. A. Lawson ssp. *orientale*), several antibacterial compounds were detected in *C. imberbe* and *C. padoides.* These results are in agreement with the antibacterial data obtained earlier.[1] A TLC survey of the *Combretum* genus indicated the presence of saponins in the leaves of *C. padoides* Eng. & Dielbs.[2] Earlier studies of *C. padoides* leaves led to the isolation of triterpenoid desmosides.[3] Other studies of the *Combretum* species resulted in the

discovery of mollic acid glycosides, anti-inflammatory and antifungal agents from *C. molle,*[4] and imberbic acid from *C. imberbe.*[5] Both types of compounds had been identified as potent molluscides. Substantial chemical work has been done on the extracts of the leaves of *C. padoides* and related species, but little has been reported on their biological activities. Investigation of 27 Combretaceae species revealed that C. *padoides* had the fifth highest antibacterial activity.[1] This paper describes the isolation, structure elucidation and some biological activities of a new triterpenoid rhamnoside and two related known compounds from *C. padoides* leaves.

Results and Discussion

Based on bioautography most of the antibacterial compounds were shown to be present in the DCM fraction. Further solvent/solvent fractionation indicated that the chloroform fraction was the most active (MIC 0.037 mg/ml). Exhaustive gradient column chromatography yielded three triterpenoids (**1-3**).

Compound **1** was isolated as white crystals and its molecular formula of $C_{38}H_{60}O_{10}$ was deduced from molecular ion peak $[M-H]^-$ at *m/z* 675.4100 in the HRESIMS (calculated 675.4108).



Compounds	R^1	R^2
1	4-Ac-O-Rh	H
2	H	OH

IR analysis indicated the presence of olefinic (1653 cm^{-1}), hydroxyl (3440 cm^{-1}), methyl (1456 cm^{-1}) and carboxylic acid (hydroxyl at 2923 cm^{-1} and 1772 cm^{1}) moieties. The triterpenoid skeleton was easily deduced from ^1H NMR data by the appearance of vinylic (R_2C=CHR, δ_H 5.24), hydroxylated methine (RCHOH, δ_H 3.55) and acetyl (CH_3COO-, δ_H 2.07) moieties (Table 1). There was also a crowded signal indicating the presence of $-CH_3$, $-CH_2$, and –CH protons (δ_H 0.5-δ_H 2.0). The ^{13}C NMR spectrum confirmed these findings. In addition it showed the typical carboxyl function at δ_c 183.1 of the $-CO_2H$ constituent. Altogether, eight methyl groups, ten methylenes, eleven methines and nine quaternary carbons were revealed by DEPT data. The ^1H

and ^{13}C NMR data of compound **1** were similar to those of **2** except for differences in signals of rings A and E relating to different hydroxylation patterns. In **1**, the methyl group at C-23 carries an ether linked substitution as indicated by the new methylene group at δ_C 70.3/δ_H 3.25 dd, 3.50 dd. In ring E, C-22 is a regular methylene and not oxygenated such as that in compound **2** (δ_c 31.1).

According to HMBC, the carboxylic acid carbonyl is located at C-20 and one of the hydroxyl groups is located at C-1 (δ_c 72.6) as earlier indicated by HMQC. Assisted by the ^{1}H-^{1}H COSY, the spin system of the complete rhamnose-skeleton (Rh') was traced. Using HMQC and ^{13}C spectra, the proton appearing as a singlet at δ_H 4.69 was assigned as an anomeric proton of the sugar unit. The proton at δ_H 4.93 (Rh'-4) was assigned as located next to an acetyl substituent of the sugar moiety. C-23 was unequivocally assigned as the site of glycosylation by a HMBC correlation between H-Rh-1 (δ_H 4.69) and the oxymethylene C-23. This position has been shown as the preferred site of glycosylation in similar compounds from the related *C. imberbe*.[5]

The relative configurations of the chiral centres at C-1, C-3, C-4, C-8, C-10, C-14, C-17, and C-20 were determined by NOESY analysis: correlations between H-1 (δ_H 3.55) and H-27 (δ_H 1.26), H-24 (δ_H 0.70) and H-25 (δ_H 1.00), H-18 (δ_H 2.01) and H-28 (δ_H 0.87), H-18 (δ_H 2.01) and H-30 (δ_H 1.19), H-Rh'-2 (δ_H 3.90) and H-Rh'-4 (δ_H 4.93), H-Rh'-1 (δ_H 3.55) and H-Rh'-4 (δ_H 4.93) indicated that these protons are oriented in the same direction. On the other hand, the NOESY correlations between H-23 (δ_H 3.50) and H-27 (δ_H 1.26), H-Rh'-6 (δ_H 1.13) and H-Rh'-3 (δ_H 4.14) indicated these to be oriented in the opposite direction. According to the coupling constants and correlation peaks in NOESY spectrum, **1** showed the same relative stereochemistry in the triterpenoid skeleton as 1α,23β-dihydroxy-12-oleanen-29-oic acid. For the stereochemistry of the sugar moiety, the signal of the anomeric carbon at δ_c 101.5 strongly suggested a β-orientation of the anomeric proton. Altogether, compound **1** was thus elucidated as 1α,23β-dihydroxy-12-oleanen-29-oic-acid-23β-O-α-4-acetylrhamnopyranoside. This compound has not been previously reported.

ESIMS analysis of **2** indicated a molecular ion peak at *m/z* 495.8 $[M+Na]^+$, suggesting a molecular formula of $C_{30}H_{48}O_4$ indicating seven degrees of unsaturation. This structural type was further supported by the ^{1}H and ^{13}C NMR spectra, which included resonances for seven skeletal methyl groups, ten methylene groups, five methyl groups, eight quaternary carbons (from DEPT) and a broad triplet at δ_H 5.25 of an olefinic proton (H-12), carboxylic acid functionality (δ_c 182.90) and two hydroxylated methines (δ_H 3.6, δ_c, 74.01 and δ_H 3.75, δc 72.97). The attribution of ^{1}H, ^{13}C, DEPT NMR data of compound **2** was in perfect agreement with those of 1,22-dihydroxy-12-oleanen-30-oic.[6] ESIMS of **3** that was isolated as greyish powder showed a molecular ion peak $[M+NH_4]^+$ at *m/z* 591.0, thus suggesting a molecular formula of $C_{35}H_{36}O_6$. Next to typical terpenoid methines and methylenes, its ^{1}H NMR spectrum revealed a sugar moiety, olefenic protons and an acetal proton. Signals for five methyls, eleven methylenes, sixteen methines, and three quaternary carbons were revealed by DEPT NMR data. ^{13}C NMR confirmed all these structural features. The structure of **3** was established as 24-ethylcholesta-7,22,25-trien-3-ol-O-β-D-glucopyranoside that has been previously isolated from *Clerodendron inerme*.[7].

Table 1. ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR data of compound **1** (CD_3OD)

Position	Carbon type	^{13}C (δ_c)	^{1}H (δ_H)[a]
1	CH	72.6	3.55 brs
2	CH_2	34.6	1.74 m, 1.93 m
3	CH	67.2	4.14 dd 13.2, 4.5
4	C	43.3	
5	CH	41.4	1.76 m
6	CH_2	18.9	1.45 m
7	CH_2	33.0	1.59 m; 1.28 m
8	C	40.8	
9	CH	39.3	2.50 dd 8.2, 8.2
10	C	42.0	
11	CH_2	24.1	1.93-1.98 m
12	CH	124.3	5.24 s
13	C	145.3	
14	C	43.5	
15	CH_2	27.4	1.86 dd 4,2, 8.0
16	CH_2	28.0	2.07 m
17	C	33.6	
18	CH	47.5	2.01 brd
19	CH_2	41.9	1.30 m; 2.21 dd 13.4, 13.4
20	C	43.6	
21	CH_2	30.4	1.87 m
22	CH_2	37.1	1.47 brt, 1.30 dd
23	CH_2	70.7	3.50 d, 9.6; 3.25 d, 9.6
24	CH_3	12.8	0.70 s
25	CH_3	17.4	1.00 s
26	CH_3	17.8	1.03 s
27	CH_3	26.8	1.26 s
28	CH_3	28.8	0.87 s
29	C	183.1	
30	CH_3	19.8	1.19 s
Rh'-1[b]	CH	101.5	4.69 brs
Rh'-2	CH	72.2	3.90 brs
Rh'-3	CH	70.7	3.91 dd cov
Rh'-4	CH	75.7	4.93 brt, 9.6
Rh'-5	CH	67.6	3.76 m
Rh'-6	CH_3	17.7	1.13 d, 10.0
Ac-1[c]	C	172.5	
Ac-2	CH_3	21.2	2.07 s

[a] multiplicity, *J* in Hz; [b] Rhamnopyranoside (Rh'); [c] acetyl (Ac)

Pentacyclic and tetracyclic triterpenes are well known for their action as molluscides particularly in their monodesmosidic form. [8] *C. molle* with its molluscidal constituent, mollic acid, has been recommended for use in rural Africa to control schistosomiasis.[9] Arjunolic acid and arjungenin, arjunglucoside pentacyclic triterpenes have been isolated from *C. molle.* [10] All compounds isolated in this study were subjected to several bioactivity assays. A serial dilution microplate assay using tetrazolium violet as growth indicator,[11] was used to evaluate the antibacterial activity of the isolated compounds (Table 2) The negative control acetone had no antibacterial activity at the 25% concentration the bacteria were subjected.

Table 2. Minimum inhibitory concentration (MIC) of isolated compounds (mg/ml)

	Minimum Inhibitory Concentration (mg/ml)			
Compound	SA	EF	EC	PA
1	0.063 ±0	> 0.25 ± 0	0.063 ±0	> 0.25 ± 0
2	0.031 ± 0	125 ± 0	0.063 ± 0	> 250± 0
3	> 0.25 ± 0	> 0.25 ± 0	0.063 ± 0	> 0.25 ± 0
Neomycin	1.9×10^{-3} ±0	1.9×10^{-3} ±0	3.9×10^{-3}	3.9×10^{-3} ±0
acetone	>25%	>25%	>25%	>25%

SA Staphylococcus aureus (ATCC 29213), *PA Pseudomonas aeruginosa* (ATCC 27853), *EC Escherichia coli* (ATCC 25922), *EF Enterococcus faecalis* (ATCC 29212).

Compounds **1** and **2** showed inhibition of the Gram-positive *S. aureus* (0.063 mg/ml, 0.031 mg/ml) and the Gram-negative *E.coli* (0.063 mg/ml for each compound), whereas *E. faecalis* and *P. aeruginosa* were resistant to all three isolated compounds, (Table 2). Compound **2** had a slightly lower MIC (0.031 mg/ml) than the active chloroform fraction (0.039 mg/ml) indicating that is not responsible for the major antibacterial activity. Compound **1** was not active at the highest level tested against the two Gram negative organisms. Compound **1** with MIC (0.063 mg/ml) greater than that of the chloroform fraction (0.039 mg/ml) may have acted in synergy with other compounds. From TLC profile examination, compound **3** was one the major compound (in terms of quantity) present in the extract.

Apart from this work, there are few data on the antimicrobial potential of the isoprenoid constituents of Combretaceae. Eloff (1998) gathered preliminary data to indicate that crude extracts of *C. imberbe and C. padoides* are active against the four most important nosocomial bacterial pathogens. Martini *et al* (2004) found good antibacterial activity of five flavonoids isolated from *C. erytrophyllum*. The antimicrobial activity of extracts of *C. erythrophyllum*,[12] may explain their use in traditional medicine for relieving symptoms that appear to be caused by infective agents e.g. bloody diarrhoea, wounds and conjunctivitis.[13]

Biological assays for antiviral, cytotoxic, and anti-proliferative activity, as well as several yeast-based target oriented assays (e.g. Myc/Max interaction, Tax/Creb interaction) did not indicate any additional biological activities of the three compounds. There was moderate enzyme

inhibition against 3α-hydroxysteroid dehydrogenase observed for **3** (data not shown) that might indicate anti-inflammatory activity. As terpenoids related to **1** and **2** from *C. imberbe*[14] showed significant inhibitory activities in this assay, an inherent anti-inflammatory activity of this class of compounds of varying intensity, though, can be assumed. Together with the non-cytotoxic effect of the compounds, this supports the use of *C. padoides* and related species in traditional medicine against infections.

Experimental Section

General Procedures. Column chromatography; silica gel 60M (230-400 mesh, Macherey-Nagel, Germany), Sephadex LH-20 (Pharmacia Biotech AB, Sweden); TLC: silica gel plates (Sil G/UV_{254}, 0.20 mm, Macherey Nagel, Germany), spots were detected at 254 or 330 nm and visualized with vanillin/H_2SO_4 spray reagent. Optical rotation, Propol digital automatic polarimeter (Dr. Wolfgang Kernchen GMBH, Germany); IR spectra, IFS55 spectrometer (Bruker, Germany); ^1H and ^{13}C NMR spectra, DPX-300, DNMR, DPX-500 (Bruker, Germany), all compounds were measured in CD_3OD or $CDCl_3$ with reference against TMS (external); ESIMS, triple quadrupole mass spectrometer Quattro (VG Biotech, England); EIMS, 70 eV direct inlet, high resolution with perfluorokerosine as a standard, MAT 95 XL (Finnegan, Germany).

Plant material. The leaves of *C. padoides* were collected from a labelled tree the Lowveld National Botanical Garden in Nelspruit, South Africa. A voucher specimen is deposited in the Botanical Garden Herbarium.

Extraction and isolation. Air-dried and powdered leaves (500 g) of *C. padoides* were defatted with hexane and extracted serially and exhaustively by maceration with dichloromethane (DCM), acetone and methanol (MeOH) to afford 12.2 g, 11.8g, 18.6 g and 97.4 g of *n*-hexane, DCM, acetone and MeOH soluble extracts, respectively. A portion (3 g) of the DCM extract, which was the most active extract according to bioautography and MIC (0.08 mg/ml), was subjected to a liquid-liquid fractionation process that afforded six fractions: chloroform (1.69g), water (0.28 g), *n*-butanol (0.78 g), hexane (0.321 g), CCl_4 (0.283 g), 65% aqueous MeOH (1.25 g). The chloroform fraction, with good antibacterial activity (MIC 0.037 mg/ml), was chromatographed on a 2x30 cm silica gel 60 gravity column using a stepwise gradient of *n*-hexane and increasing amounts of ethyl acetate (EtOAc) (20%, 800 ml each step), followed by EtOAc with increasing amounts of MeOH (10% 800 ml each step) ending at 50% MeOH. Collected fractions were evaporated under vacuum and examined by TLC analysis. Similar fractions were pooled to give seven major fractions (F_1-F_7) all exhibiting antibacterial activity. Crystals were observed in a tube containing fraction F_4 and were washed with a gradient of hexane and acetone to yield 7 mg of compound **2** with an R_f value of 0.5 in

ethylacetate/methanol/water = 40:5.4:4 (EMW) as solvent system at 25 ^{0}C. A large quantity of sediment was filtered from fraction F_6 and washed with 100% hexane, followed by hexane/chloroform mixtures with increasing amounts of chloroform (10% 400 ml at each step, up to 50%) to give 53 mg of compound **3** with an R_f value of 0.3 in EMW at 25 ° C. A 150 mg of fraction F_7 was further chromatographed on a 3 x 120 cm Sephadex LH-20 with methanol (1000 ml) as eluent. This resulted in 5 mg of **1** with an R_f value of 0.22 in EMW as solvent system at 25 ° C.

1α,23β-Dihydroxy-12-oleanen-29-oic-acid-23β-O-α-L-4-acetylrhamnopyranoside (1). White crystals, $[\alpha]_D^{26}$ +31.39° (*c* 0.15, MeOH); IR (film): ν_{max} = 3440, 2923, 1772, 1653, 1456 cm^{-1}. ESIMS 1370.8 $[2M+NH_4]^+$, 694.8 $[M+NH_4]^+$. HRESIMS 675.4100 calculated 675.4108) $[M-H]^-$. ^{1}H and ^{13}C NMR: see Table 1.

1,22-Dihydroxy-12-oleanen-30-oic acid (2). Mp, optical rotation, EIMS, IR and ^{13}C NMR data were identical to that reported in the literature. [6]

24-Ethylcholesta-7,22,25-trien-O-β-D-glucopyranoside (3). Mp, optical rotation, EIMS, IR and ^{13}C NMR data were identical to that reported in the literature**.** [7]

Biological Activity

Bioautography. Bioautography,[15] was used in the bioassay-guided isolation of antibacterial compounds and fractions. Developed chromatography plates of 10 mg/ml extracts and fractions were dried over night and sprayed with a suspension of growing cells of Gram-positive or Gram-negative bacteria, and incubated at 37 ^{0}C in a chamber at 100% relative humidity for 18 hours. After spraying with *p*-iodonitrotetrazolium salt, clear zones on the chromatogram indicated inhibition of growth after incubating for 1 hour at 37 ^{0}C.

Microplate dilution. A 2-fold serial dilution microplate method [11] was used to determine the minimum inhibitory concentrations (MIC) of fractions and pure compounds against *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922. Tetrazolium violet was used as an indicator of growth.

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References

1. Eloff, J. N. *South African Journal of Science* **1999**, *5*,148.
2. Carr, J. D.; Rogers, C. B. *South African Journal of Botany* **1997**, *53,*173.
3. Rogers, C. B. *Phytochemistry* **1988**, *27*, 3217.
4. Pegel, K. H.; Rogers, C. B. *J. Chem. Soc., Perkin Trans.* **1985**, *1*, 1711.
5. Roger, C. B.; Subramony, G. *Phytochemistry* **1988**, *27*, 531.
6. Nakano, K.; Oase, Y.; Takaishi, Y. *Phytochemistry* **1997**, *46*, 1179.
7. Atta-Ur-Rahman, S.; Sumayya, S.; Iqbal, M.; Farzana, A. *Phytochemistry* **1997**, *46*, 1721.
8. Marston, B.; Hostettmann, K. *Phytochemistry* **1985**, *24*, 639.
9. Rogers, C. B. *Phytochemistry* **1995**, *40*, 833.
10. Panzini, I.; Verotta, L.; Rogers, C. B. *South African Journal Science* **1993**, *89*, 324.
11. Eloff, J. N. *Planta Medica* **1998**, *64*, 711.
12. Martini, N. D.; Eloff, J. N. *Journal of Ethnopharmacolgy* **1998**, *62*, 255.
13. Gelfand, M.; Mavi, S.; Drummond, R.; Ndemera, B; The traditional medical practitioner in Zimbabwe, first ed. Mambo press Gweru, **1985**, 256.
14. Angeh J. E.; Huang X.; Sattler I.; Swan G. E.; Dahse H.; Härtl A.; Eloff J. N. *Journal of Ethnopharmacology* In press **2007** http://dx.doi.org/, doi:10.1016/j.jep2006.09.002
15. Begue, W.; Kline R. *Journal of Chromatography* **1972**, *64,* 182.