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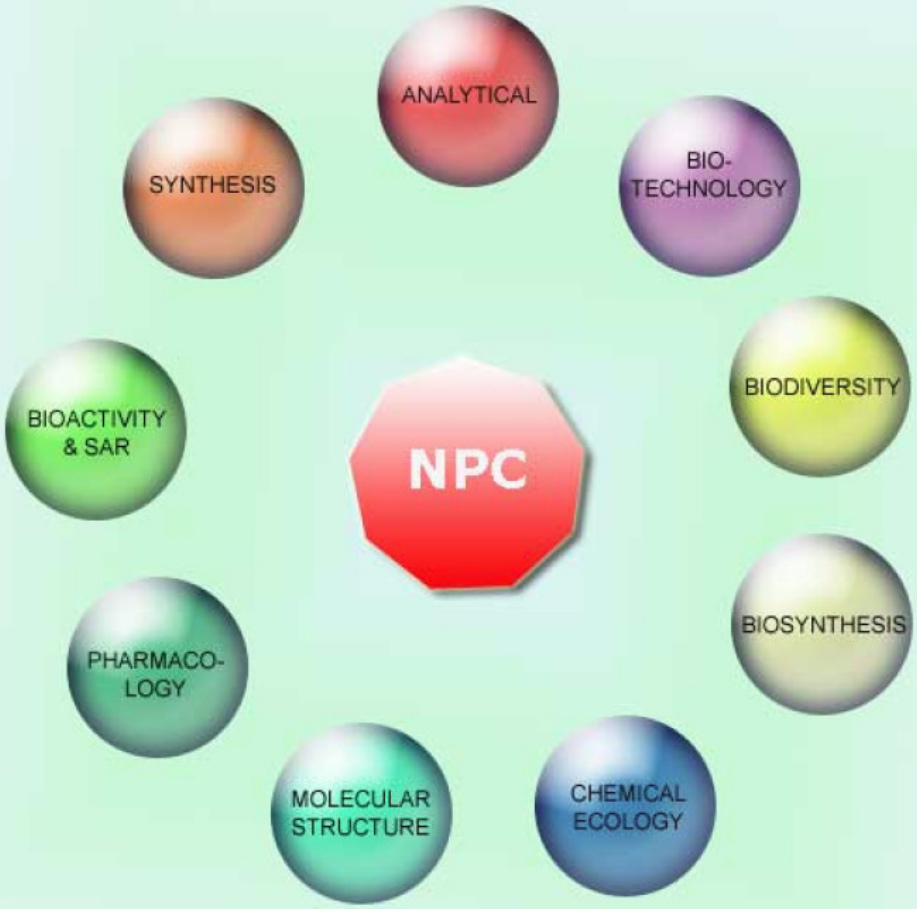

This Issue is Dedicated to
Professor Peter G. Waterman

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Antibacterial Diterpenes from the Roots of *Ceriops tagal*

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Dedicated to Professor Peter G Waterman, one of the pioneers of phytochemical research.

Investigation of the roots of *Ceriops tagal* led to the isolation of a new isopimarane, together with the known diterpenes isopimar-8(14)-en-15,16-diol and erythroxyl-4(17),15(16)-dien-3-one. The structure of the new compound was identified as isopimar-8(14)-en-16-hydroxy-15-one. These structures were determined from extensive spectroscopic data analysis. The isolates were screened for antibacterial activity using the agar dilution method against ten test bacterial strains (*Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus kristinae*, *Pseudomonas aeruginosa*, *Salmonella pooni*, *Serratia marcescens*, *Staphylococcus aureus*, *S. epidermidis* and *Streptococcus pyrogens*). Isopimar-8(14)-en-16-hydroxy-15-one exhibited activity, with MIC values of 0.5 mg/mL against *Streptococcus pyrogens*; 0.25 mg/mL against *Salmonella pooni* and 0.1 mg/mL against *Bacillus cereus*, *Staphylococcus aureus* and *Micrococcus kristinae*.

Keywords: *Ceriops tagal*, Rhizophoraceae, diterpenes, antibacterial activity.

The genus *Ceriops* (Rhizophoraeae) is represented by two mangrove plant species, *C. tagal* (Perr.) C.B. Robinson and *C. decandra* (Griff.) Ding Hou [1]. They are widely distributed along the sea coasts of Africa, South Asia and the South Pacific islands [2]. *C. decandra* has for many years been used for the treatment of malaria, malignant ulcers, hemorrhages, infected wounds and diabetes [2-5]. *C. tagal* is used for the treatment of diarrhea, vomiting, amoebasiasis and ulcers [1]. The stems, twigs, hypocotyls and fruits of *C. tagal* have been studied and the ethyl acetate extracts of the stems and twigs yielded dolabrane-type diterpenes and a norditerpene [6], while the hypocotyls and fruits yielded dammarane, lupane and oleanane-type triterpenes [7]. In this study, the chloroform extract of the roots was investigated and resulted in the isolation of a new isopimarane, along with two known diterpenes. In this paper, the isolation, structure elucidation and antibacterial activity of the reported compounds are discussed.

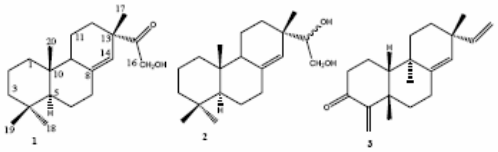

Figure1: Structures of compounds **1**, **2** and **3**.

Air dried roots of *C. tagal* were extracted with chloroform at room temperature and the extract filtered and concentrated. The crude extract was fractionated through silica gel column chromatography to afford a series of fractions, which on further purification, afforded a new diterpene isopimar-8(14)-en-16-hydroxy-15-one **1**, together with two known diterpenes, isopimar-8(14)-en-15,16-diol **2**, and erythroxyl-4(17),15(16)-diene-3-one **3**. Compound **2** was first reported from the leaves of *Isodon flavidus* (Lamiaceae) and named glavidusin A [8]. Compound **3** was recently reported from the stems and twigs of *C. tagal* [6], but compound **2** is reported for the first time from the genus *Ceriops*.

Compound **1** was isolated as colorless crystals with a melting point of 76-78°C. Its HR-MS showed a molecular ion peak at *m/z* 304.2148 $[M]^+$, consistent with the molecular formula $C_{20}H_{32}O_2$. The IR spectrum showed absorption bands at 3200, 1720 and 1620 cm^{-1} suggesting the presence of hydroxyl, carbonyl and olefinic groups, respectively. The information from the ^{13}C NMR (Table 2) and DEPT spectra indicated the presence of five quaternary carbons (δ_C 214.7, 143.0, 46.7, 38.5 and 33.2), three methine carbons (δ_C 122.9, 54.6 and 51.0), eight methylene carbons (δ_C 65.8, 42.0, 38.8, 35.8, 32.8, 22.4, 20.1 and 18.8) and four methyl carbons (δ_C 33.6, 27.3, 22.0 and 14.4) suggesting a diterpenoid skeleton. The ^1H NMR spectrum (Table 1) showed the presence of four methyl singlet signals (δ_H 1.09, 0.85, 0.80 and 0.62), hydroxymethyl protons (δ_H 4.33, 1H, *d*, *J* = 1.2 Hz and δ_H 4.31, 1H, *d*, *J* = 1.5 Hz), an olefinic proton (δ_H 5.34, 1H, *dd*, *J* = 3.9, 1.5 Hz), and a number of overlapping methylene proton signals. The hydroxymethyl protons (δ_H 4.33 and δ_H 4.31) showed HMBC correlation with a carbonyl carbon (δ_C 214.7) and a quartenary carbon (δ_C 46.7), suggesting the existence of the partial structure [-C-CO-CH_2OH or –CO-C-CH_2OH] in compound **1**. The existence of a former partial structure was confirmed by a fragment ion peak at *m/z* 245 (M-59) in the ESI-MS spectrum corresponding to the loss of a –CO-CH_2OH fragment in the mass spectrum. The data above suggested that compound **1** possessed an isopimarane skeleton with a hydroxyethanone side chain.

The ^{13}C NMR spectroscopic data of compound **1** were similar to those of compound **2**. Close examination of ^1H and ^{13}C NMR spectral data of these two compounds revealed that the difference between them is the presence of a keto group (C-15, δ_C 214.7) in compound **1** instead of a hydroxymethyl group (C-15, δ_C 78.7) in the latter. The olefinic carbons (δ_C 143.0 and δ_C 122.9) were assigned to C-8 and C-14, respectively, following the HMBC correlations between the olefinic proton resonating at δ_H 5.34 (δ_C 122.9) and carbons resonating at δ_C 35.8 (C-7), 51.0 (C-10), 33.2 (C-12), 214.7 (C-15) and a methyl carbon at δ_C 27.3 (C-17). The remaining methyl groups were assigned to C-18 (δ_H 0.85; δ_C 33.6), C-19 (δ_H 0.80; δ_C 22.0) and C-20 (δ_H 0.62; δ_C 14.4), based on HMBC data analysis. The stereochemistry of compound **1** was established by NOESY experiments. The NOESY spectrum (Figure 2) showed a correlation between Me-20 and both Me-19 and H-11β, as well as between Me-17 and H-11β,which indicated that the configuration of Me-17, 19 and 20 were β. Correlation between Me-18 and H-5 was also evident in the NOESY spectrum (Figure 2) confirming the α orientation of Me-18 and H-5.

Table 1: ^1H (300 MHz) NMR chemical shifts for compound **1** in $CDCl_3$.

Position	^1H (δ)	Position	^1H (δ)
1a	0.94, dd, (12.6, 4.5)	11a	1.07-1.14
1b	1.52-1.64	11b	1.52-1.64
2a	1.35-1.44	12a	2.27, ddd (14.1, 12.6, 4.8)
2b	1.52-1.64	12b	2.30, ddd (14.1, 5.8, 3.3)
3a	1.07-1.14	14	5.34, dd (3.9, 1.5)
3b	1.35-1.44	16a	4.31, d (1.2)
5	1.02, dd (12.6, 2.4)	16b	4.33, d (1.2)
6a	1.31, ddd (12.6, 4.8, 2.1)	17	1.09, s
6b	1.52-1.64	18	0.85, s
7a	2.05, ddd (14.3, 12.9, 5.8)	19	0.80, s
7b	2.31, ddd (14.3, 4.4, 2.1)	20	0.62, s
9	1.72, t (8.1)		

Assignments were confirmed by COSY, HMQC, HMBC and DEPT experiments. *J* values, in Hz,, are in brackets.

Table 2: ^{13}C (75.4 MHz) NMR chemical shifts for compound **1** in $CDCl_3$.

Position	^{13}C (δ)	Position	^{13}C (δ)
1	38.8 (t)	11	20.1 (t)
2	18.8 (t)	12	32.8 (t)
3	42.0 (t)	13	46.7 (s)
4	33.2 (s)	14	122.9 (d)
5	54.6 (d)	15	214.7 (s)
6	22.4 (t)	16	65.8 (t)
7	35.8 (t)	17	27.3 (q)
8	143.0 (s)	18	33.6 (q)
9	51.0 (d)	19	22.0 (q)
10	38.5 (s)	20	14.4 (q)

Assignments were confirmed by COSY, HMQC, HMBC and DEPT experiments

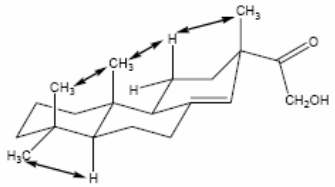

Figure 2: Key NOE correlations of compound **1**.

Moreover, the assignment of the olefinic and methylene carbons was in agreement with the reported data for compound **2** [8]. Compound **1** was, therefore, identified as isopimar-8(14)-en-16-hydroxy-15-one. Compound **2** is a precursor of compound **1** from a biosynthetic point of view.

The antibacterial effect of the reported compounds was tested against five Gram-positive and five Gram-negative bacterial strains. Generally, the activity displayed was very weak compared to the standard.

Table 3: Antibacterial activity of **1**, **2** and **3** from the roots of *Ceriops tagal*.

Bacterial strains	Compounds MIC (mg/mL)[a]			
	1	2	3	C
Gram positive bacteria				
Bacillus cereus	0.1	0.5[c]	0.25	+
Staphylococcus aureus	0.1	0.5	0.25	+
Micrococcus kristinae	0.1	na	na	+
Staphylococcus epidermidis	na[d]	na	na	+
Streptococcus pyrogens	0.5	na	na	+
Gram negative bacteria				
Escherichia coli	na	na	na	+
Salmonella pooni	0.25	na	na	+
Serratia marcescens	na	na	na	+
Pseudomonas aeruginosa	na	na	na	+
Klebsiella pneumoniae	na	na	na	

[a]Minimum inhibitory concentration. C = Chlorampenicol, used as control at 0.001 mg/mL.
[c]Maximum concentration of the compounds tested. [d]Not active.

However, isopimar-8(14)-en-16-hydroxy-15-one (**1**) was the most active (Table 3), followed by erythroxyl-4(17),15(16)-diene-3-one (**3**) and isopimar-8(14)-en-15,16-diol (**2**). The compounds had no significant activity against the Gram-negative bacteria, with the exception of *Salmonella pooni*, which was inhibited by **1** at 0.25 mg/mL. Compounds **1** and **2**, both of which are isopimaranes, differ only in that the former has a keto group at C-15, while the latter has a hydroxymethine group at that position. The difference in their antimicrobial potencies indicates that a keto group at C-15 may be important for the antibacterial activity of isopimaranes.

Experimental

General experimental procedures: Mps uncorrected: Stuart Scientific melting point apparatus. IR: Perkin-Elmer 2000 FT-IR spectrometer. ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC and HMBC were acquired on Bruker Avance DPX 300 Spectrometer using standard pulse sequences and referenced to the residual solvent signal. LR-MS were obtained on a Finnigan MAT LCQ^{DECA} instrument, and HR-MS were obtained on a GCT Premier instrument. The UV and visible (UV-VIS) spectra were taken on a Shimadzu UV-2101PC UV-Vis Scanning Spectrophotometer. IR spectra were measured on a Perkin Elmer System 2000 FT-IR Spectrophotometer, using KBr pellets. Specific rotations $[\alpha]_D$ were measured on a polatronic-D (Schmidt + Haensch) polarimeter. Analytical TLCs were prepared on ready made 0.25 mm layers of Merck silica gel 60 $F_{254+366}$ coated aluminium foil. Spots on the chromatograms were detected by observing in UV light (254 or 366 nm) and / or by spraying with vanillin-sulfuric acid spray. Prep. TLCs were prepared on 0.5 mm thick layers of Merck silica gel 60 $HF_{254+366}$ containing $CaSO_4$ (binder) coated on 20×20 cm glass plates. Column chromatography was conducted using different sizes of columns packed with Merck silica gel 60, particle size 0.0400-0.0630 mm and Sephadex LH-20.

Plant material: Roots of *Ceriops tagal* were collected from Maruhubi Mangrove Reserve, Zanzibar, Tanzania in November 2004. The plant material was authenticated by Mr Mtumwa, Institute of Marine Sciences, University of Dar es Salaam. Voucher specimen, coded CT 03 2005, was deposited in the Institute of Marine Sciences, University of Dar es Salaam.

Extraction and isolation: The air dried roots of *C. tagal* (740.5 g) were pulverized and shaken in chloroform at room temperature for 24 h before the extract was concentrated under reduced pressure to afford 120.5 g brown extract. The obtained extract was adsorbed onto 150 g silica gel and applied to a silica gel column packed with 1200 g of silica gel, using $CHCl_3$. The column was eluted using $CHCl_3$ [fractions 1-22] and $CHCl_3$/acetone (1:1) [fractions 23-25]. Based on the TLC analysis, these fractions were combined as follows: A (fractions 1-19), B (fractions 20-22), and C (fractions 23-25). The combined fraction B (20.6 g) was adsorbed on silica gel, packed onto a silica gel column, and eluted with *n*-hexane/acetone (10:1) to give fractions B1 and B2. Fraction B1 was left in a conical flask for 36 h; colorless crystals formed, which were recrystallized in methanol to give isopimar-8(14)-en-15,16-diol (**2**) (46.8 mg) [8]. The combined fraction C (15.4 g) was adsorbed on silica gel, packed onto a silica gel column, and eluted with *n*-hexane/acetone (10:2) to give fractions C1 and C2. Fractions C1 and C2 were left overnight and colorless crystals were observed; these were recrystallized in methanol and identified as isopimar-8(14)-en-16-hydroxy-15-one (**1**) (10.3 mg) and erythroxyl-4(17),15(16)-dien-3-one (**3**) (39.5 mg) [6], respectively.

Isopimar-8(14)-en-16-hydroxy-15-one (1)

White crystals.
MP: 76-78°C.
$[\alpha]_D$: +28.65° (*c* 1.00, $CHCl_3$).
IR (KBr pellets) ν_{max}: 1620 (C=C), 1720 (C=O), 3200 (=CH) cm^{-1}.
^{1}H NMR: Table 1.

^{13}C NMR: Table 2.
ESI-MS *m/z* (rel. int.): 305 $[M + H]^+$ (25), 289 (20) and 245 (100); HR-MS *m/z* 304.2148 $[M]^+$ calculated for $C_{20}H_{32}O_2$.

Antibacterial assays: Adopting the method of Afolayan and Meyer [9], nutrient agar (NA) for bacteria was prepared and autoclaved before the compounds were added. To test at 0.5 mg/mL, 5 mg of compound was dissolved in 0.1 mL of acetone and added to 9.9 mL of molten nutrient medium. The mixture was poured into a Petri dish, swirled carefully until the agar began to set and left overnight for the solvent to evaporate. Laboratory isolates of ten bacterial strains were used, which included five Gram positive and five Gram negative (Table 3) obtained from the Department of Microbiology, University of Rhodes. Each organism was maintained on NA slants (Biolab) and was recovered by culturing in nutrient broth No. 2 (Biolab) for 24 h at 37°C and each culture was diluted 1:100 with fresh sterile nutrient broth. The organisms were streaked in radial patterns on agar plates [9], incubated at 30°C, and examined after 24 and 48 h. Complete growth inhibition by a specific concentration was required for it to be declared active. The tested concentrations were 0.5, 0.25, 0.1 and 0.05 mg/mL, chloramphenicol was used as a standard control, and blank plates containing either nutrient agar only or nutrient agar and 1% acetone, without the compound, served as blank controls. Each treatment was done in triplicate.

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