Benthaminin 3, a Novel Antibacterial Cassane-Type Furanoditerpenoid from *Caesalpinia benthamiana*

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Abstract: The roots of *Caesalpinia benthamiana* (*synonym. Mezoneuron benthamianum*) are considered to be an effective remedy in Ghana for the management of skin diseases and wounds. Bioactivity-guided fractionation of the chloroform extract of the root bark of the plant has resulted in the isolation of a novel cassane-type furanoditerpenoid, designated as benthaminin 3. The structure of the compound was elucidated by the use of spectroscopic techniques. The antibacterial activity of the compound has been assessed using the microdilution assay method. The lowest MIC (63µg/mL) was recorded against *Staphylococcus aureus* and *Bacillus subtilis.* The compound was also observed to have a mild inhibitory effect against resistant strains of bacteria including methicillin-resistant *S. aureus,* tetracyline-resistant *S. aureus and* erythromycin-resistant *S. aureus* with MIC values greater than 1000µg/mL.

Keywords: *Caesalpinia benthamiana* (Baill.) Herend. and Zarucchi*, Mezoneuron benthamianum* Baill, Caesalpinaceae, Cassane-type furanoditerpenoids, Antibacterial activities.

1. INTRODUCTION

Caesalpinia benthamiana (Baill.) Herend. and Zarucchi (*syn. Mezoneuron benthamianum* Baill.) (Caesalpinaceae) [1] is a shrub found mostly in secondary forest in Ghana and finds use in the treatment of topical infections and wounds [2,3]. Traditionally, a paste made from powdered root bark mixed with shea butter, palm oil or palm kennel oil may be used topically [4]. Plants belonging to the genus *Caesalpinia* are a rich source of cassane-type furanoditerpenoids which are reported to possess a number of biological activities e.g. antiplasmodial [5], antiviral [6] and anti-diarrhoeal effects [7].

In a previous study of this species we reported the isolation and characterization of two new cassane diterpenoids, benthaminins 1 and 2, from the active fractions of the petroleum spirit extract possessing antibacterial and antioxidant activities [8]. A third compound was also isolated which was a deoxy form of caesaldekarin C (also referred to as methyl vouacapenate) which had previously been isolated from *Caesalpinia major*, *C. bonducella, Vouacapoua americana and V. macropetala* [9,10].

In a continuation of this research the antibacterial properties of the chloroform extract of the root bark of *C. benthamianum* were investigated using antimicrobial assay procedures.

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2. RESULTS AND DISCUSSION

2.1. Isolation and Purification of Compound RC4 (Designated Benthaminin 3)

Bioactivity-guided analysis was carried out using agar overlay bioautography on six fractions (MCF1-MCF6) obtained from silica gel CC of the chloroform extract of *C. benthamiana*. MCF2 and MCF6 were found to be active against *B. subtilis*.

Further fractionation of MCF6 on column chromatography (SG/DCM:MeOH) yielded fractions MCF21-MCF25.

MCF25 was subjected to further repeated column chromatography on silica to afford a pure compound RC4, eventually designated benthaminin 3 (33mg). The structure of the compound was elucidated using a combination of 1D and 2D NMR spectroscopy, mass spectrometry (LREIMS, HREIMS and ESI) and reference to the literature [8, 9, 11].

2.1.1. Compound RC4

Benthaminin **3** (**RC4)** was obtained as white amorphous compound; LREIMS *m/z* 384. See Table **1** for ^{1}H and ^{13}C NMR data. It had molecular formula $C_{23}H_{28}O_5$ based on HREIMS spectral analysis of *m/z* 384. The ^{1}H NMR spectrum (Table **1**) is similar to that of deoxycaesaldekarin C previously isolated from the petroleum spirit extract of the same plant [8, 9, 11] apart from the presence of an additional acetyl group shown by the methyl signal at δ 2.33 and the fact that

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Table 1: ^{1}H NMR and ^{13}C NMR Spectroscopic Data (δ, Hz) of Benthaminin 3 in $CDCl_3$

Position	^{1}H Chemical shifts (ppm)	^{13}C Chemical shifts (ppm)
1α	1.56 (*m*)	36.5
1β	1.77 (*m*)	
2α	1.57 (*m*)	18.1
2β	1.65(*m*)	
3α	1.29 (*m*)	38.4
3β	1.74(*m*)	
4		48.0
5	1.90 (*m*)	42.1
6	5.30 (*s*)	70.7
7α	2.95 (*m*)	21.7
7β	2.99 (*m*)	
8		128.7
9		145.5
10		38.0
11	6.72 (*s*)	105.0
12		153.8
13		123.8
14		125.8
15	6.73 (*s*)	106.6
16	7.26 (*s*)	144.5
17		24.3
18	1.45 (*m*)	16.5
19		178.6
20	1.25 (*m*)	16.0
21	3.72 (*s*)	52.3
22		170.8
23	2.33 (*s*)	18.9

ring C was aromatic as shown by carbon signals at δc 128.7(C-8), 145.5 (C-9), 105.0 (C-11), 153.8 (C-12), 123.8 (C-13), 125.8 (C-14), 106.6 (C-15) and 144.5 (C-16). RC4 also showed resonances for the 2,3-disubstituted furan at δ7.26 (*s*) and δ6.73 (*s*), downfield from the corresponding positions in the spectrum of deoxycaesaldekarin C, indicating a more electronegative environment. This was explained by the fact that the adjacent ring was aromatic in nature, as shown by the shifts of the relevant C atoms δ128.7(C-8), δ 145.5 (C-9), δ 105.0 (C-11), δ 153.8 (C-12), δ 123.8 (C-13) andδ 125.8 (C-14), similar to those shown by benthaminin 1 isolated previously [8]. This was confirmed by HMBC studies which showed the aromatic proton at δ6.73 was directly attached to a carbon resonating at δ105.0 (assigned to C-11). This proton also showed HMBC correlations to aromatic carbons at δ123.8 (C-13), δ128.7 (C-8) and a quaternary carbon at δ38.0 (C-10). The presence of an additional aromatic singlet at δ6.73 (H-15) and a methyl attached to an aromatic ring (δ1.45) confirmed the presence of an aromatic ring of a caesaldekarin type. Two 3H singlets appeared at δ3.72 and δ2.33 indicated the presence of a methoxy ester and acetyl groups. These data are broadly consistent with the structure being an aromatized form of deoxycaesaldekarin C, analogous to benthaminin 1 but with an additional acetyl group. The additional acetyl group is considered to be attached at C-6 (δc 70.8), with the 6-H resonating at δ5.3, which is similar to the signal observed in caesalmin C [6]. The methyl group signal at δ1.25 had HMBC correlations to the ester carbonyl signal at δ178.6 (C-19), the quaternary carbon at δ 48.0 (C-4) and a methylene carbon at δ38.4 (assigned to C-3).

NOESY correlations are as in caesalmin C and deoxycaesaldekarin C [6, 8].

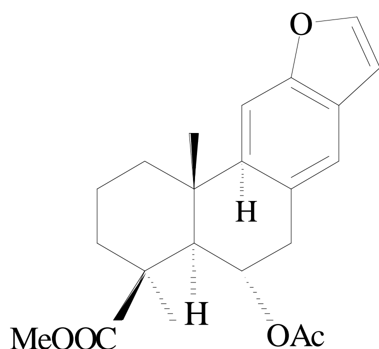

Figure 1: Benthaminin 3.

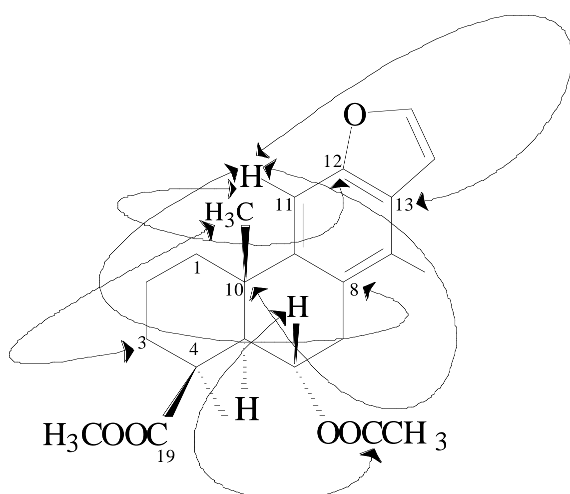

Figure 2: HMBC correlations for benthaminin 3.

2.1.2. Antibacterial Effects of Benthaminin 3

Bioactivity studies of the compound revealed that it possessed antibacterial activity against ten Gram positive and Gram negative bacteria, including three resistant strains namely, methicillin-resistant *S. aureus,* tetracyline-resistant *S. aureus and* erythromycin-resistant *S. aureus.* In all cases with MIC values were greater than 1000µg/mL (Table **2**). Benthaminin 3 was however more active against *Staphylococcus aureus* and *Bacillus subtilis* with an MIC of 63µg/mL (Table **2**). Microbial infections and the presence of oxygen free radicals are known impediments to wound healing [12]. Notable among the micro-organisms delaying or inhibiting wound healing are *Staphylococcus, Streptococcus* and *Pseudomonas* species [13]. On the basis of the antibacterial properties demonstrated by benthaminin **3** as well as other antibacterial and antioxidant cassane-type diterpenoid compounds isolated from the petroleum spirit extract of the root bark of this plant [8], the traditional use of the lipophilic preparations of these species is supported.

3. CONCLUSION

In the current study, a novel compound designated benthaminin 3, a cassane-type furanoditerpenoid compound possessing antibacterial activity has been isolated from the chloroformic extract of *C. benthamiana*. To the best of our knowledge, this represents the first report of the isolation of this compound from *C. benthamiana*.

4. EXPERIMENTAL

4.1. Materials, Methods and Techniques

4.1.1. Plant Materials

The root bark of *C. benthamiana* (Baill.) Herend. and Zarucchi was collected from Akwapim Mampong in

Table 2: Minimum Inhibitory Concentration (MIC) (µg/mL) of Compound on Test Organisms

Test Organism	Strain	Benthaminin 3
Methicillin-resistant *S. aureus*	SA 1199B (NorA)	>1000
Tetracycline-resistant *S. aureus*	XU 212 (Tek)	>1000
Erythromycin-resistant *S. aureus*	RN 4220 (MsrA)	>1000
S. aureus (Wild)	NCTC 4263	63
B. subtilis	NCTC 10073	63
S. faecalis	NCTC 775	125
P. aeruginosa	NCIMB 1042	250
M. flavus	NCTC 7743	125
M. fortuitum	ATCC 6841	250
M. smegmatis	ATCC 14468	>1000

All experiments were carried out in triplicate. 200µg/mL of tetracycline and rifampicin served as positive controls (MIC = 20µg/mL); n = 3.

the Eastern region of Ghana in August, 2003. It was authenticated by Mr H R Blagogee, a taxonomist at the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong Akwapim, Ghana, where voucher specimen #RADMB11 is deposited.

4.1.2. Extraction and Isolation

2.7Kg (dry weight) of coarsely powdered root bark of *C. benthamiana* were extracted with chloroform (5L) using a Soxhlet apparatus for 24h. The extract was concentrated under reduced pressure at 40°C to dryness to obtain a dark-brown syrup [20.9g (1.1%) yield]. This extract was subjected to VLC on silica gel 1000g, eluted initially with chloroform (2L) and subsequently with chloroform:MeOH 95:5 (6L), 9:1 (9L), 4:1 (4L), 3:2 (5L), 2:3 (2L) and finally with MeOH (3L). The TLC profiles [Give TLC system used] of aliquots collected enabled six major fractions to be bulked to give MCF1 (1.3g), MCF2 (2.8g), MCF3 (9.70g), MCF4 (0.97g), MCF5 (2.63g) and MCF6 (2.05g). These were tested for antibacterial activity (The lowest MIC values for the various fraction were as follows: MCF1 (>1000 µg/mL against all test organisms), MCF2 (125 µg/mL against *S. aureus*), MCF3 (>1000 µg/mL against all test organisms), MCF4 (250 µg/mL against *B. subtilis* and *M. flavus*), MCF5 (>1000 µg/mL against all test organisms) and MCF6 (250 µg/mL against *B. subtilis*, *M. flavus* and *S. faecalis*)) and the most active fraction MCF2 was further fractionated using CC SG/ chloroform:MeOH gradient to yield fractions MCF21-MCF25. MCF25 was subjected to column chromatography on silica gel (400g), eluting with chloroform and gradually increasing the polarity of the solvent by 1% increments of MeOH. Aliquots were monitored on TLC and fractions showing a single zone were combined and pure compound RC4, later designated benthaminin **3**, isolated after a final preparative TLC separation.

4.1.3. Chromatographic Investigation

4.1.3.1. Analytical TLC

Precoated aluminium-backed silica Gel F_{254} TLC plates (0.25mm thickness), product code, OB481662, were purchased from Merck KGaA, Germany. Suitable solvent systems were used and acidic anisaldehyde used as detecting reagent.

4.1.3.2. Preparative TLC (PrepTLC)

The plates were prepared by mixing 90g of silica gel 60 PF_{254}, product code, TA 1349747 525, for preparative thin-layer chromatography (Merck KGaA, Germany), with 260 ml of distilled water. The slurry was spread onto 5 glass plates with the aid of a spreader to give 1.0 mm thickness. After drying at room temperature, the plates were activated at 105°C for 2 hours. Zones were detected under UV 254nm as quenching zones, or showed florescence at 366nm, scraped off, eluted with the solvent system: chloroform: petroleum spirit (7:3), filtered and the resulting solutions were evaporated to dryness under reduced pressure. Analytical TLC was then used to check the level of purity of the compounds. Impure samples were subjected to further preparative TLC analysis until a pure sample was obtained.

4.1.3.3. Vacuum Liquid Chromatography (VLC)

The dry method of packing was used in this instance. The column was packed by putting silica gel 60 (0.040-063mm), product code TA 1280185 438, for column chromatography (Merck KGaA, Germany) in a Buchner funnel attached to a 1000L conical flask with a side arm connected to a vacuum supply. The sample was triturated with an equal weight of silica gel 60 (4.2g and 40.9g for preliminary and bulk experiments respectively), and applied to the top of the column. A thin film of non absorbent cotton wool was spread evenly on top of the column to prevent disturbance to the surface of the column when the eluant was being added. The eluant was added at a fixed volume of 50mL and 200mL respectively for both preliminary and bulk experiments, gradually and carefully. Vacuum was then applied and the eluant was sucked through the column by reduced pressure into the collecting conical flask. Fractions (between 100-200mL) were collected as aliquots of eluants of one polarity. Low polarity eluants were used initially and the polarity gradually increased. At any given polarity, the eluant was continually added and aliquots collected until no compounds were being desorbed from the column into the collectors, this being detected by TLC monitoring. After elution of compounds ceased, the polarity of the eluant was increased and the entire process repeated until finally 100% of the most polar solvent was used to wash the column. For the preliminary VLC analysis, a smaller Buchner funnel (10cm x 8cm), was employed using 400g of silica gel. The bulk of the VLC was done using 1500g of silica gel in a bigger Buchner funnel (25cm x 20cm). In all cases, based on the TLC profiles of aliquots collected, similar fractions were bulked and the solvents recovered using a rotary evaporator under reduced pressure. Bioactivity studies were carried out on all fractions to determine which fractions possessed activity. All active fractions were further subjected to column chromatography (CC) separations, and other techniques, to isolate the active compounds

responsible for any observed activity. Since VLC is a quick and crude way of dividing the crude extracts into relatively smaller units, larger aliquot volumes are collected, unlike in CC, where aliquots collected are in relatively small volumes.

4.1.3.4. Column Chromatography

The wet method of packing was employed here. Column was packed with silica gel 60 (0.040-0.063mm), product code TA1280185438, for Column Chromatography (Merck KGaA, Germany), wetted with the initial eluting solvent of the lowest polarity. The sample was triturated with equal amount of silica gel and applied to the top of the column. After the sample was adsorbed on top of the column, eluants of increasing polarities were added in fixed volumes. Fractions were eluted from the column under gravity and collected in glass specimen tubes. As in VLC, the TLC profiles of aliquots collected were monitored by analytical TLC, by observing under UV at both 254 and 366nm and then spraying with acidic anisaldehyde reagent.

4.1.4. Determination of Minimum Inhibitory Concentration (MIC)

Stock solutions were prepared by dissolving 4mg RC4 of the compound in 80µl of DMSO. Sterile water was added with slight heating to aid dissolution and the volume made up to 2ml in a sterile bottle. This was then sonicated to ensure complete dissolution. The stock mixture was passed through a 0.2µm pyrogenic filter to sterilize the solution and serially diluted to arrive at concentrations between 1000µg/ml and 7.8µg/ml. The inocula of micro-organisms were prepared from broth cultures, and serial dilutions were made to achieve a suspension of approximately 10^5CFU/ml. For every experiment, a sterility check (2% DMSO and medium), negative control (2% DMSO, medium and inoculum), and positive control (2% DMSO, medium, inoculum and water-soluble antibiotic) were included. The 96-well plates were prepared by dispensing into each well 100µL each of double strength nutrient broth, 100µL of test solutions and 20µl of the inoculum. Contents of each well were thoroughly mixed with a sterile-tipped multi-channel pipette and the plates incubated at 37^oC for 24 hours. Any growth of the micro-organisms was determined by adding 20µl of a 5% solution of tetrazolium salt (MTT) and incubating for a further 30 minutes. Dark coloured wells indicated growth whilst colourless wells indicated inhibition of growth of organisms. 200µg/ml tetracycline served as a positive control. All experiments were performed in triplicate.

4.1.5. Micro-Organisms Used

The following bacterial strains obtained from the UK National Culture Collection and School of Pharmacy, University of London, were used in the bioassay: *Micrococcus flavus* (NCTC 7743), *Bacillus subtilis* (NCTC 10073), *Staphylococcus aureus* (NCTC 4163), *Mycobacterium fortuitum* (ATCC 6841), *Mycobacterium smegmatis* (ATCC 14468), multidrug-resistant *S. aureus* SA-1199B, tetracycline-resistant *S. aureus* XU 212, erythromycin-resistant *S. aureus* RN 4220, *Streptococcus faecalis* (NCTC 775) and *Pseudomonas aeruginosa* (NCIMB 10421).

5. AUTHENTIC REFERENCE

5.1. Antibiotics

Tetracycline and rifampicin were obtained from Sigma Aldrich Co Ltd. UK

5.2. Chemicals

Unless otherwise specified, chemicals were analytical grade and purchased from Sigma Aldrich Co Ltd. UK. Organic solvents were analytical grade and purchased from BDH Laboratory Supplies, UK.

5.3. Apparatus

Low resolution ESI mass spectra were run on a QToF (Waters) instrument. The NMR spectra were obtained on a Bruker 500 spectrometer with chemical shifts reported in δ (ppm) using TMS as an internal standard.

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