Four new *neo*-clerodane diterpenes from the stem bark of *Croton oligandrus*

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ABSTRACT

Four new *neo*-clerodanes, crotonolins C-F (**3-6**), were isolated from the stem bark of *Croton oligandrus* together with the known clerodane crotonzambefuran A, the abietanes 7-*β*-hydroxydehydroabietic acid and 7-oxodehydroabietic acid, and ferulic acid. Their structures were elucidated by spectroscopic analyses including 1D and 2D NMR and HRESIMS and by comparison with previously reported data. The cytotoxicity of the isolated compounds against A549, MCF7, PC3 and PNT2 cells was evaluated using the MTT assay. Only 7-*β*-hydroxydehydroabietic acid showed a moderate level of activity against PC3 cells with an IC_{50} value of 68.9 ± 6.6 µM.

KEYWORDS

Croton oligandrus; Euphorbiaceae; *neo*-clerodane; crotonolins, cytotoxicity

1. Introduction

The genus *Croton* L. (Euphorbiaceae) is well known for producing clerodane diterpenes (Amaral and Barnes, 1998; Salatino *et al*., 2007; Xu *et al*., 2018), which are a group of bicyclic diterpenes widespread in the family Euphorbiaceae and known for their wide range of biological activities including anticancer, anti-inflammatory, antifeedant and anti-ulcer among others (Li *et al*., 2016). *Croton oligandrus* Pierre ex Hutch is a small aromatic tree growing up to 5 - 10 m tall, commonly found in Western and Central African forests (Aubreville, 1983). In Cameroon, it is found in the Central, Southern and Western regions and is used traditionally as a remedy for the treatment of anaemia, pneumonia and splenomegaly (Aubreville, 1983; Jiofack *et al*., 2009; Betti *et al*., 2013). In our previous investigation (Guetchueng *et al*., 2018), we reported five clerodanes: 12-*epi*-megalocarpoloide D, crotocorylifuran, crotonolins A and B and megalocarpoidolide D along with two triterpenes, acetyl aleuritolic acid and lupeol and three phenolic compounds, cluytyl ferulate, hexacosanoyl ferulate and vanillin. In continuation of our study on the diterpenoids of *C. oligandrus*, we have investigated the minor constituents of the plants and characterised four new *neo*-clerodanes, for which we have given the trivial name crotonolins C-F (**3**-**6**).

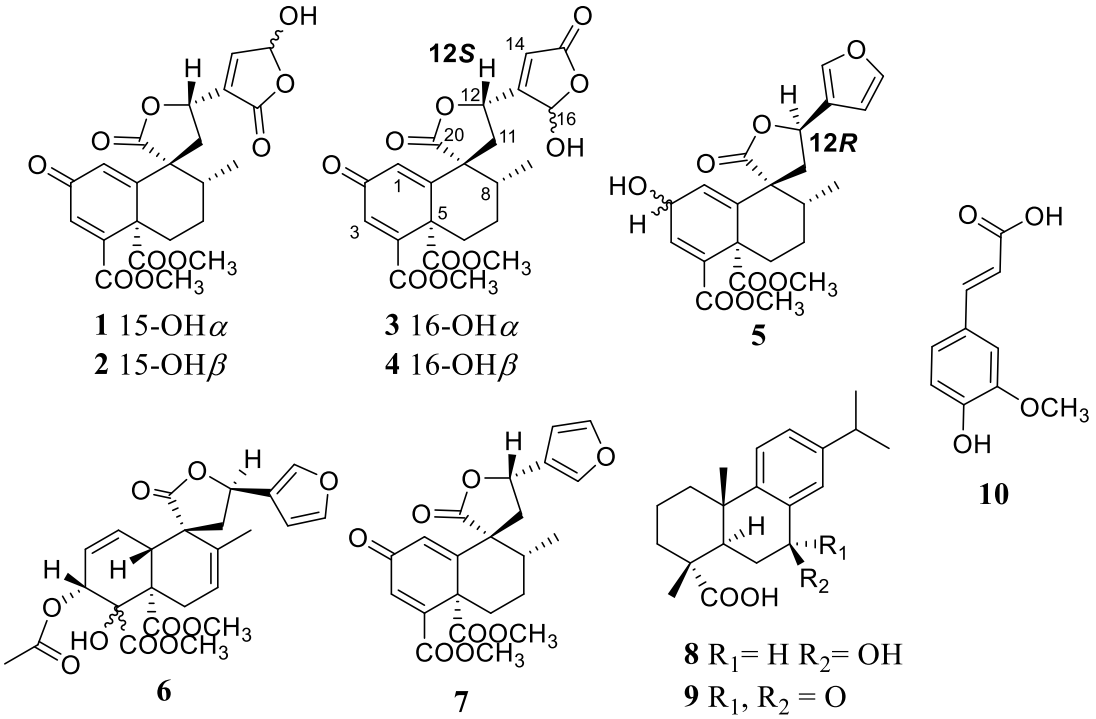

Figure 1. Structures of compounds **1**-**10** isolated from the stem bark of *C. oligandrus*

2. Results and discussion

Reversed-phase HPLC separation of the methanolic extract of *C. oligandrus* yielded, in addition to the previously reported compounds (Guetchueng *et al.*, 2018), four new *neo*-clerodanes, named crotonolins C-F (**3**-**6**) along with the known clerodane crotonzambefuran A (**7**) (Ngadjui *et al*., 2002), the abietanes 7-*β*-hydroxydehydroabietic acid (**8**) and 7-oxodehydroabietic acid (**9**) (Tanaka *et al.*, 1997) and ferulic acid (**10**) (Nguyen *et al*., 2004) (Figure 1). The known compounds (**7**-**10**) were identified by comparison of their spectroscopic data with their corresponding available data in the literature.

Compounds **3** and **4** were isolated as a white amorphous powder mixture whose molecular formulae $C_{22}H_{22}O_{10}$ were determined by HRESIMS by the peak at *m/z* 469.1120 $[M + Na]^+$, calculated for $C_{22}H_{22}O_{10}Na^+$, 469.1105. Their FTIR spectrum showed absorption bands of hydroxy (3465 cm^{-1}), *ɤ*-lactone (1775 cm^{-1}), *α*, *β*-unsaturated lactone (1712 cm^{-1}) and olefinic (1670 cm^{-1}) groups. Their ^1H and ^{13}C NMR data rather similar to those of clerodanes crotonolins A and B (**1**-**2**), previously isolated from the same plant (Guetchueng *et al*., 2018) suggested that these compounds were structurally close. Complete assignment of rings A and B and their respective substituents could be achieved easily by comparison of the observed chemical shifts of **3** and **4** with those of crotonolins A and B (**1**-**2**). These assignments were further supported by the different correlations observed in the HMBC experiment (Supplementary data). The spectroscopy data of compounds **3** and **4** mainly differed from those of **1** and **2** by the signals attributable to the modified furan ring on position C-12. It was clear that instead of the 15-hydroxy-13-en-15,16-olide moieties present in crotonolins A and B, the C-12 substituents in the case of compounds **3** and **4** were identified as 16-hydroxy-13-en-15,16-olides (Blas *et al.*, 2004; Shirota *et al.*, 2006; Maldonado *et al.*, 2016). Combined analysis of the different signals and the correlations observed in the 1D and 2D NMR spectra of **3** and **4** confirmed the suggested structure elements. The resonances at δ_C 163.6 could be attributed to the *β*-carbon (C-13), δ_H 6.22 and δ_C 119.2 to the *α*-methine (C-14), δ_C 169.1 to the lactone

carbonyl (C-15), and δ_H 6.28 and δ_C 96.7 to the hemiacetalic methine (C-16). The relative configuration of **3** and **4** were determined using NOESY (Supplementary data) except for the hemiacetalic proton H-16, for which no conclusive correlation was observed. The NOESY spectrum supported the 12*S* configuration of both compounds as strong correlations were observed between H-12 and H-1 (Ndunda *et al*., 2016). Therefore, compounds **3** and **4** were characterized as 2,15-dioxo-16*β*-hydroxy-18,19-dimethoxycarbonyl-15,16-epoxy-*ent*-cleroda-1(10),3,13(16),14-tetraen-20,12-olide and 2,15-dioxo-16*α*-hydroxy-18,19-dimethoxycarbonyl-15,16-epoxy-*ent*-cleroda-1(10),3,13(16),14-tetraen-20,12-olide, respectively, and were given the trivial names crotonolins C (**3**) and D (**4**), respectively.

Compound **5** was isolated as a white amorphous powder. Its molecular formula $C_{22}H_{24}O_8$ was determined from the ion peak at *m/z* 417.1555 $[M+H]^+$ calculated for $C_{22}H_{24}O_8H^+$, 417.1543 from its HRESIMS spectrum obtained in positive ion mode. Its ^{1}H NMR displayed characteristic signals corresponding to those of a secondary methyl at δ_H 1.17 (d, *J* = 6.7 Hz); a *β*-substituted furan ring at δ_H 6.44 (d, *J* = 0.9 Hz), 7.48 (brs) and 7.49 (dd, *J* = 0.9, 1.6 Hz); four methines including two olefinic signals at δ_H 6.04 (brt, *J* = 1.9 Hz) and 6.70 (dd, *J* = 1.9, 2.4 Hz) and two oxymethines at δ_H 4.87 (t, *J* = 2.4 Hz) and 5.53 (dd, *J* = 6.4, 8.7 Hz). The observed chemical shifts were similar to those observed for megalocarpoidolide D (Ndunda *et al*., 2016; Guetchueng *et al.*, 2018) with the only major difference being the presence of an additional oxymethine signal at δ_H 4.87 (t, *J* = 2.4 Hz) on the ^{1}H NMR spectrum of **5**, but absent on that of megalocarpoidolide D. This observation was confirmed by the correlation observed in the HSQC-DEPT spectrum of **5** between this proton and the carbon signal at δ_C 64.4. In the ^{1}H-^{1}H COSY spectrum of **5** correlations could be observed between the two olefinic protons at δ_H 6.04 (brt, *J* = 1.9 Hz, H-1) and 6.70 (dd, *J* = 1.9, 2.4 Hz, H-3) and the oxymethine at δ_H 4.87 (t, *J* = 2.4 Hz) suggesting the reduction of the carbonyl function present in megalocarpoidolide D on position C-2 (Ndunda *et al*., 2016; Guetchueng *et al.*, 2018) to an alcohol function in **5**.

Furthermore, in the HMBC experiment (Supplementary data), correlations could be observed between the proton signal at δ_H 4.87 (H-2) and the carbons at δ_C 129.4 (C-1) and 136.2 (C-3); the signal at δ_H 6.04 (H-1) and the carbon signals at δ_C 48.3 (C-5), 53.3 (C-9) and 136.2 (C-3), as well as the correlations of the proton signal at δ_H 6.70 (C-3) and the carbons at δ_C 48.3 (C-5), 129.4 (C-1) and 166.2 (C-18). In the NOESY spectrum, a strong correlation observed between the 3H-17 and H-12 suggested a 12*R* configuration of C-12. None of the observed correlations was helpful in determining the 2-OH orientation. Thus, all the observed spectroscopic data led to the identification of compound **5** as 2-hydroxy-18,19-dimethoxycarbonyl-15,16-epoxy-*ent*-cleroda- 1(10),3,13(16),14-tetraen-20,12-olide and was named crotonolin E.

Compound **6** was isolated as a white amorphous powder. Its molecular formula $C_{24}H_{26}O_{10}$ was determined from its HRESIMS spectrum obtained in the positive ion mode where the peak at *m/z* 497.1445 $[M+Na]^+$ was calculated for $C_{24}H_{26}O_{10}Na^+$, 497.1418. Compound **6** could be identified as a *β*-substituted furan clerodane from its 1D and 2D NMR which showed some similar features to compounds **5**. The ^{1}H NMR of **6** showed a couple of overlapping signals for the olefinic protons at δ_H 5.88 (H-2) and 5.89 (H-1), which showed scalar coupling in the COSY spectrum with an oxymethine proton at δ_H 5.21 (H-3, brs) as well as with a methine at δ_H 3.14 (H-10, brs). In addition, the presence in the COSY spectrum of correlations between H-3 and H-10 suggested the presence of an homoallylic coupling system. A vinyl methyl proton resonance at δ_H 1.84 (3H, t, J = 1.2 Hz, H-17), which showed

correlations in the HMBC spectrum with the olefinic carbons at δ_C 126.0 (C-7) and 129.3 (C-8) and the quaternary carbon at δ_C 51.9 (C-9) was also observed. The ^{13}C NMR revealed characteristic signals of *γ*-lactone (δ_C 175.7, C-20; 71.8, C-12) and a *β*-substituted furan moiety (δ_C 124.8, C-13; 108.1, C-14; 144.2, C-15; 139.6, C-16). The signals at δ_C 172.0, 170.1 and 170.7 were attributed to the carbonyls of two methyl esters (C-18 and C-19) and an acetoxy

function respectively. In the HMBC spectrum, ^{3}J long-range ^{1}H-^{13}C correlations could be observed between the oxymethine proton at δ_H 5.21 (H-3) and the carbonyl of the acetoxy at δ_C 170.7 suggesting an acetylation on that position C-1. Correlations were also observed between the carbon δ_C 77.9 (C-4) and the protons at δ_H 5.21 (H-3), 3.14 (H-10) and 2.36 (H-6). Hence, A-ring of compound **6** was identified as a 3-acetoxy-1-ene. In the NOESY spectrum, a strong correlation observed between H-12 and 3H-17 suggested a 12*R* configuration of C-12. The correlation observed between H11*pro-R*(δH 2.43)/H10 established the *β*-orientation of H-10. In addition, the appearance of proton signals of H-3 (δ_H 5.21) and H-10 (δ_H 3.14) as broad singlets also indicating the *β*-orientation of H-3. The NOESY experiment was not helpful to determine the position of the 4-OH groups. Based on the observed data, the structure of compound **6** was elucidated as 3*α*-acetoxy-4-hydroxy-18,19-dimethoxycarbonyl-15,16-epoxy-*ent*-cleroda-1,7,13(16),14-tetraen-20,12-olide, a 1,2- dehydro derivative of the previously reported compound megalocarpoidolide H isolated from *Croton megalocarpoides* (Ndunda *et al*., 2016), a new *neo*-clerodane diterpene and given the trivial name crotonolin F.

Compounds **3 - 4** and **6 - 10** were evaluated for their cytotoxicity against A549, MCF7, PC3 and PNT2 cells. The concentrations inhibiting fifty percent (IC_{50}) of cell growth after treatment of each compound were determined. All the isolated compounds failed to demonstrate a significant toxicity against the tested cell lines. Only 7-*β*-hydroxydehydroabietic acid (**8**) was found to have a moderate effect on the PC3 cells (IC_{50} 68.9 ± 6.6 µM).

3. Experimental

3.1. General experimental procedures

Chromatographic solvents were purchased from Fisher Scientific, UK, and used without further purification. NMR spectroscopic analyses were performed on a Bruker AMX 600 NMR spectrometer (600 MHz for ^{1}H, and 150 MHz for ^{13}C). MS analyses were performed on a Xevo G2-S ASAP or LTQ Orbitrap XL 1 spectrometers. HPLC-DAD analyses were performed on Agilent 1260 Infinity series. Extracts and fractions were analyzed on a Phenomenex Gemini-NX 5 U C_{18} column (150 × 4.6 mm, Phenomenex, USA) or ACE Gemini-NX 5 U C_{18} column

(150 × 21.2 mm, Hichrom Ltd, UK) with a gradient 30-100% MeOH (0.1% TFA), 70-0% H_2O (0.1% TFA) over 30 min. The column temperature was set at 25°C. The chromatogram was monitored at 1 mL/min or 10 mL/min using variable UV–vis wavelengths (215, 254, 280 and 320 nm). Optical rotation was determined using Bellingham-Stanley ADP660 polarimeter (MeOH, c in g/100mL). IR were recorded on an Agilent Cary 630 FT-IR.

3.2. Plant material

The bark of *Croton oligandrus* Pierre ex Hutch. was collected at Mount Eloundem, Centre Region, Cameroon, in June 2015, and identified by Mr Victor Nana, a retired taxonomist at the Cameroon National Herbarium, where a voucher specimen (6687/SFR) was deposited.

3.3. Extraction and isolation

The air-dried and ground bark (350.0 g) of *C. oligandrus* was successively extracted with *n*-hexane, DCM and MeOH using a Soxhlet extractor (800 mL, 10 cycles each). After evaporation at 40^{o}C under reduced pressure, 4.1 g, 2.9 g and 7.8 g of *n*-hexane, DCM and MeOH extracts, respectively, were obtained. The DCM extract was cleaned up using solid phase extraction as follow. The extract was suspended in 10 mL of 20 % MeOH - water and loaded on to a 20 g Strata C-18-E cartridge (Phenomenex, USA), previously washed with MeOH (50 mL), followed by equilibration with water (100 mL). The cartridge was eluted with 100 mL MeOH-water 50 %. The resultant solution was concentrated to dryness using a rotary evaporator under reduced pressure to yield 1.8 g of extract. The obtained extract was resuspended in MeOH to obtain a 53.5 mg/mL solution and subjected to preparative HPLC using an ACE prep-column (150×21.2 mm, Hichrom Ltd, UK), flow rate 10 mL/min, mobile phase gradient of water (A) and methanol (B) both containing 0.1% TFA: 30-60% B, 0-15 min, 60-100% B 15-35 min monitored at wavelength 215 nm to yield the mixture of **3** and **4** (6.5 mg), **5** (2.9 mg), **6** (7.3 mg), **7** (3.3 mg), **8** (5.7 mg), **9** (4.1 mg) and **10** (8.3 mg) having retention times 19.55, 15.76, 16.19, 29.29, 30.31, 23.03 and 12.51 min, respectively.

3.4. Spectroscopic data

*Crotonolins C (**3**)* and *D (**4**)*, white amorphous powder mixture. (6.5 mg). $[\alpha]_D^{25}$ -16.5 (c 0.002, MeOH). HRESIMS *m/z* 469.1120 $[M+Na]^+$, calculated for $C_{22}H_{22}O_{10}Na^+$, 469.1105 FT-IR (ATR) ν_{max} 3465, 2950, 1775, 1712 and 1670 cm^{-1}. ^1H and ^{13}C NMR data (See Tables S1 and S2)

*Crotonolin E (**5**)*, white amorphous powder (2.9 mg). $[\alpha]_D^{25}$ -70.0 (c 0.005, MeOH). HRESIMS *m/z* 417.1555 $[M+H]^+$ calculated for $C_{22}H_{24}O_8H^+$, 417.1544. FT-IR (ATR) ν_{max} 3350, 2950, 1775, 1712 and 1510 cm^{-1}. ^1H and ^{13}C NMR data (See Tables S1 and S2)

*Crotonolin F (**6**)*, white amorphous powder (7.3 mg). $[\alpha]_D^{25}$ +58.4 (c 0.003, MeOH). HRESIMS *m/z* 497.1445 $[M+Na]^+$, calculated for $C_{24}H_{26}O_{10}Na^+$, 497.1418. FT-IR (ATR) ν_{max} 3510, 2950, 1715 and 1610 cm^{-1}. ^1H and ^{13}C NMR data (See Tables S1 and S2).

3.5. In vitro cytotoxic assessment

To assess the cytotoxicity of the crude extracts, fractions and isolated compounds from *C. oligandrus*, the following cell lines were used: A549 (adenocarcinoma human alveolar basal epithelial cell line), MCF7 (human breast adenocarcinoma cell line), PC3 (human prostate cancer cell line) and PNT2 (human normal prostate epithelium cell line). The cells were grown in RPMI-1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% foetal bovine serum (FBS) and cultured at 37°C, 5% CO_2 and 95% humidity. For experimental use, the cells were seeded into 96 well plates (1.2×10^4/well) and incubated for 24 h. Cells were then treated with crude extract (0-250 µg/mL) for 24 h or isolated compounds (0 to 200 µM) for 48 h and the cell viability measured using the MTT assay (Mossman *et al.*, 1983). The formazan crystals formed were dissolved in DMSO and optical density was read at 570 nm using a ClarioStar microplate reader (BMG Labtech, UK). Three individual wells were assayed per treatment; the assay was repeated three times and cytotoxic activity was determined using the percentage absorbance compared to control cells [(absorbance of treated cells/absorbance of untreated cells) × 100]. Doxorubicin was used as positive control and the IC_{50} value of each test sample was calculated using the software Graphad Prism 7.02.

4. Conclusion

Four new neo-clerodanes (**3**-**6**) together with four known compounds (**7**-**10**) were isolated from the stem bark of *C. oligandrus* (Euphorbiaceae). This study has provided relevant information on the phytochemicals present in *C. oligandrus* and confirms the richness of the *Croton* genus in clerodane diterpenes as well as their structural diversity. None of the isolated compounds has demonstrated a significant cytotoxic effect against the cell-lines tested.

Disclosure statement

The authors declare no conflict of interest.

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