Diterpenoids from the Roots of *Croton dichogamus* Pax

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ABSTRACT:

Four previously undescribed diterpenoids including two crotofolanes, crotodichogamoin A and B, and two halimanes, crothalimene A and B, a new sesquiterpenoid, and fifteen previously reported compounds, including the crotofolane, crotohaumanoxide, the casbane, depressin, a further seven furanohalimane diterpenoids, three patchoulane and two further cadinane sesquiterpenoids and aleuritolic acid were isolated from the root of *Croton dichogamus*. Crotodichogamoin B is an important biosynthetic intermediate of the crotofolane class and this is the first report of patchoulene sesquiterpenoids from the genus. Compounds were tested at one concentration, $1x10^{-5}$ M, in the NCI59 cell one-dose screen but did not show significant activity snd were also evaluated for their cytotoxicity against Caco-2 cell lines using the neutral red assay. 10-*epi*-Maninsigin D reduced Caco-2 cell viability at 10, 30 and 100 µM, with values of decreased viability of 28%, 48% and 43% respectively. None of the other tested compounds showed significant activity.

Keywords:

Croton dichogamus, Euphorbiaceae, crotofolane, crotodichogamoin B, crothalimene A, crothalimene B, patchoulane, Caco-2 cell viability

1. Introduction

The genus *Croton* is one of the largest of the Euphorbiaceae family with more than 1200 species occurring in the tropics and subtropics worldwide (Berry et al., 2005). Several species of *Croton* are used in South America, Asia and Africa in traditional medicine to treat abdominal pain, abscesses, dyspepsia, gastric and duodenal ulcers, impetigo, (Giang et al., 2003), diabetes, diarrhoea, and as an analgesic (Suárez et al., 2003), to treat dysmenorrhoea (Thongtan et al., 2003), respiratory diseases (Aguilar-Guadarrama and Rios, 2004), rheumatoid arthritis, snake bites, stomach aches, jaundice (Huang et al., 2015), wounds (Lopes et al., 2004; Salatino et al., 2007), as a tonic and to improve digestion (Asare et al., 2011), and as a mild laxative (Ngadjui et al., 1999). The sap of *Croton lechleri* Müll. Arg., known as "dragon's blood", is used as a household remedy to treat diarrhoea, stomach ulcers, pain and swelling of insect bites and herpes infections (Jones, 2003, Gupta et al., 2008; Sollmann, 1920). Phytochemical studies of *Croton* have shown the presence of alkaloids, phenolics, terpenoids and volatile oils, with a wide range of diterpenoid classes predominating (Salatino et al., 2007). Compounds from the *Croton* genus have been widely screened and have shown anti-angiogenic (Huang et al., 2015), antibacterial (Bayor et al., 2009), anti-inflammatory (Aguilar-Guadarrama and Rios, 2004), antinociceptive, (Suárez et al.,), antimalarial and antimycobacterial activity, and to show cytotoxicity towards Vero, KB and BC cell lines (Thongtan et al., 2003), to inhibit LPS-Induced NF-KB activation (Giang et al., 2003) and to be cytotoxic against human cervical carcinoma cells (Block et al., 2002).

Croton dichogamus Pax (Euphorbiaceae) grows as a shrub or tree in Ethiopia, Kenya, Madagascar, Mozambique, Tanzania and Somalia (Govaerts, 1999). This species is used as a dietary additive to milk or meat soup by the Maasai and Batemi of Kenya and Tanzania respectively. It is known as "Mhand" in Tanzania, where it is used to treat respiratory diseases by inhalation of smoke of the plant leaves (Mohagheghzadeh et al., 1999). It is used to treat chest complaints, malaria and stomach upsets in the Samburu region of Kenya, where it is commonly known as "l-akirding'ai" (Fratkin, 1996). In the Loitoktok district of Kenya, it is known as "Oloibor benek", and is used to treat arthritis and gonorrhoea (Muthee et al., 2011). A previous investigation of the leaves of *C. dichogamus*, collected in Kenya, led to the isolation two crotofolane diterpenoids, crotoxide A and B (Jogia et al., 1999). In the present study, the root gave five previously unreported compounds, crotodichogamoin A **(2)** and B **(3)**, 15,16-epoxy-3-hydroxy-5(10), 13(16), 14-*ent*-halimatriene-17, 12(*S*)-olide **(5)**, 15,16-epoxy-5,13(16),14-*ent*-halimatriene-3-ol **(6)**, and 1,3,5-cadinatriene-(7*R*),(10*S*)-diol

(19) together with 15 previously reported terpenoids. We report the structures of these five compounds, the previously unreported NMR data of the known compound 15,16-epoxy-4(18),13(16),14-*ent*-clerodatrien-3α-ol (gbaninol) **(11)**, along with the an evaluation of the cytotoxicity of compounds tested.

2. Results and discussion

The *n*-hexane, CH_2Cl_2 and MeOH extracts of the root of *Croton dichogamus* were separated using flash chromatography followed by preparative thin layer chromatography and/or gravity column chromatography to yield five previously unreported (**2**, **3**, **5**, **6**, and **19**) and 15 known compounds. The known compounds were identified from their spectroscopic data and by comparison against literature data, as crotohaumanoxide (**1**) previously isolated from *Croton haumanianus* (Tchissambou et al., 1990), depressin (**4**), a casbain diterpenoid previously isolated from the soft coral *Sinularia depressa* (Li et al., 2010), 15,16-epoxy-3α,4β-dihydroxy-13(16),14-*ent*-clerodadien-17,(12*S*)-olide (furocrotinsulolide A) (**7**) previously isolated from *Croton insularis* (Graikou et al., 2005), 15,16-epoxy-3-keto-13(16),14-*ent*-clerodadien-17,(12*S*)-olide (crotonolide E) (**8**) and 15,16-epoxy-3α-hydroxy-4(18),13(16),14-*ent*-clerodatrien-17,(12*S*)-olide (crotonolide F) (**9**) previously isolated from *Croton laui* (Liu et at., 2014), 3β,4β,15,16-diepoxy-13(16),14-*ent*-clerodadiene-17,(12*S*)-olide (**10**) previously isolated from *Croton oblongifolius* (Pudhom and Sommit; 2011), 15,16-epoxy-4(18),13(16),14-*ent*-clerodatrien-3α-ol (gbaninol) (**11**), 15,16-epoxy-13(16),14-*ent*-clerodadien-3-one (*trans*-cascarillone) (**12**) previously isolated from *Croton sonderianus* (McChesny and Silveira, 1990), 3β,4β,15,16-diepoxy-13(16),14-clerodadiene (**13**) previously isolated from *Thysananthus spathulistipus* (Harinantenaina et al., 2006), 4-patchoulene (cyperene) (**14**) and 4-patchoulen-3-one (cyperotundone) (**15**) previously isolated from *Cyperus rotundus* (Joseph-Nathan et al., 1984; Xu et al., 2009), 6α-methoxypatchoulan-4-ene (**16**) previously isolated from *Croton muscicapa* (Barreto et al., 2013), 4-isopropyl-1,6-dimethylnaphthalene (cadalene) (**17**) previously isolated from *Siparuna macrotepala* (El-Seedi, 1994), 1(6),7,9-cadinatriene-4α,5β-diol (4α,5β-corocalanediol) (**18**) previously isolated from *Juniperus formosana* (Kuo et al., 1990), and aleuritolic acid (**20**) previously isolated from many sources, including *C. pseudopulchellus* (Langat et al., 2012). The structures of the patchoulenes (**14-16)** were determined with the assistance of the NMR Predict programme (Robien, 2014). This is the first report of patchoulene sesquiterpenoids from *Croton*. Gbaninol (**11**) has been isolated previously from *Gossweilerodendron*

balsamiferum Harms (Caesalpiniaceae) (Ekong and Okogun, 1969), however, no NMR data has been reported for this compound.

The NMR spectra of compound **2** showed similarities to those of the co-isolated crotofolane, crotohaumanoxide (**1**) (Tchissambou et al., 1990). HREIMS data showed an $[M+H]^+$ ion at *m/z* 327.1590 indicating a molecular formula of $C_{20}H_{22}O_4$. The FTIR spectrum showed absorption bands at 1747 cm^{-1} for a carbonyl stretch, 1649 cm^{-1} for a double bond stretch, and 1269 cm^{-1} for an epoxide C-O stretch. The ^{13}C NMR spectrum showed twenty carbon resonances, which could be assigned using HSQC, COSY and HMBC experiments and by comparison with NMR data for crotohaumanoxide (**1**). The C-1β acetate group present in compound **1** was missing and a ketone group (δ_C 206.1) occurred at this position in compound **2**. The presence of a furan ring was shown by resonances at δ_C 117.0 (C-15), δ_C 121.8 (C-14), δ_C 137.0 (C-16) and δ_C 150.4 (C-13) and the C-14, C-15 and C-16 resonances showed correlations in the HMBC spectrum with the 3H-17 resonance (δ_H 1.96). The two H-12 resonances (δ_H 2.81, 2.95) showed correlations in the HMBC spectrum with the C-10 (δ_C 141.9), C-11 (δ_C 37.8), C-13 and C-14 resonances and correlations in the COSY spectrum with two H-11 resonances (δ_H 2.53, 2.78). A pair of exomethylene proton resonances (δ_H 5.06, 4.67, bs, 2 H-18) showed correlations in the HMBC spectrum with the C-8 (δ_C 39.8), C-10 and C-11 resonances, confirming its placement at C-10. The C-18 resonance (δ_C 116.9) showed correlations with the H-8 and two H-11 resonances, and the H-8 resonance showed further correlations with the C-1 (δ_C 206.1), C-4 (δ_C 65.8), C-6 (δ_C 61.5), C-7 (δ_C 49.0), C-9 (δ_C 66.1), C-10 and C-14 resonances and a correlation in the COSY spectrum with the H-7 resonance (δ_H 2.49, d, J=13.0 Hz). The H-7 resonance showed correlations in the HMBC spectrum with the C-5 (δ_C 56.2), C-6, C-8, C-9, C-10, C-13, C-14, C-15 and C-20 resonances. The chemical shifts of the C-9/C-4 and C-5/C-6 resonances indicated the presence of epoxides at these positions as in crotohaumanoxide. The H-5 resonance occurred as a singlet (δ_H 3.29) and its assignment was confirmed by correlations with the C-4, C-6, C-9 and methyl C-20 (δ_C 8.9) resonances. Both the C-4, C-9 and ketone carbon resonance showed correlations in the HMBC spectrum with the H-2 (δ_H 2.49) and two H-3 (δ_H 2.66 and 1.84) proton resonances. The 3H-19 resonance (δ_H 1.11, d) showed correlations in the HMBC spectrum with the C-2, C-3 and the ketone carbon resonance, confirming the placement of the keto group at C-1. The COSY spectrum showed coupling between the 3H-19/H-2 and H-2/two H-3 resonances. The relative configuration was assigned using the NOESY spectrum. Correlations were seen between the H-5/3H-20, 3H-

20/H-8 and H-8/3H-17 resonances, and, therefore, they were assigned as β as in crotohaumanoxide (**1**). Configurations at C-4 and C-9 could not be confirmed from the NOESY spectrum, but it was assumed that they would be the same as in the co-isolated crotohaumanoxide and the related crotocascarin A (Kawakami et al., 2013) with both the epoxide groups on the α-face. In both crotohaumanoxide and crotocascarin A the relative configuration was confirmed by X-ray analysis.

Compound **3**, crotodichogamoin B, isolated as a yellow oil from the CH_2Cl_2 extract, was found to be an interesting crotofolane diterpenoid and a possible parent to the crotofolane class. The HRMS data showed a $[M+H]^+$ ion at *m/z* 295.1694 indicating a molecular formula of $C_{20}H_{22}O_2$ and ten degrees of unsaturation. The FTIR spectrum showed a strong carbonyl stretch band at 1730 cm^{-1}. The ^{13}C NMR spectrum showed twenty carbon resonances, including two ketone group carbon resonances at δ_C 208.1 (C-1) and δ_C 202.5 (C-13). Resonances due to furan ring carbons, present in **1** and **2**, were not present. Vinyl methyl groups occurred at C-16 (δ_C 23.5) and C-17 (δ_C 22.1) and the HMBC spectrum showed correlations between the corresponding 3H-16 (δ_H 1.49), 3H-17 (δ_H 2.13) and C-15 (δ_C 147.5) and C-14 (δ_C 134.3) alkene carbon resonances. The HMBC spectrum showed correlations between the C-14 and C-13 (δ_C 202.5) ketone carbonyl carbon resonance and the two H-12 resonances (δ_H 2.27 and δ_H 2.43). The C-13 resonance also showed a correlation with the two H-11 resonances (δ_H 2.82 and δ_H 2.72), which showed correlations with the C-10 (δ_C 142.0) and C-18 (δ_C 114.8) resonances of the exomethylene group and the C-8 (139.2) aromatic ring carbon resonance. The 3H-20 resonance showed correlations with C-5 methine (δ_C 126.9) and C-6 (δ_C 137.9) and C-7 (δ_C 143.4) fully substituted carbon resonances. The corresponding H-5 singlet resonance (δ_H 7.22) showed correlations with the C-3 (δ_C 34.4), C-4 (δ_C 131.0), C-6 (δ_C 137.9), C-7 (δ_C 143.4), C-9 (δ_C 153.3) and C-20 (δ_C 20.6) resonances. The C-1 (δ_C 208.0) ketone carbonyl resonance showed correlations with the two H-3 (δ_H 2.26, 3.34), the H-2 (δ_H 2.72) and 3H-19 (δ_H 1.28, d, *J*=7.1) proton resonances in the HMBC spectrum. The H-2 resonance showed correlations with the C-4 (δ_C 131.0) and C-9 (δ_C 153.5) resonances. The configuration at C-2 in compound **3** was assumed to be the same as in compounds **1** and **2.**

It has been proposed that crotofolones arise from geranylgeraniol cyclising to form cembranoids, from which casbenes, such as **4** co-isolated in this work, form. Bond formation between C4/C-9 gives the lathrane class, and further C-7/C-8 bond formation leads to the

jatrophanes. Cleavage of the C-13/C-15 bond leads to the formation of the crotofolanes (Kawakami et al., 2013). Oxidation to a keto-group at C-13 and cyclisation with C-16 leads to the formation of a furan ring. Intermediates to the furan ring formation have been reported from *C. inularis* (Maslovskaya et al., 2014).

Compound **5**, 15,16-epoxy-3β-hydroxy-5(10),13(16),14-*ent*-halimatriene-17,(12*S*)-olide, isolated as a white solid from the CH_2Cl_2 extract, was found to be a previously undescribed furanohalimane diterpenoid. The HREIMS data showed a $[M]^+$ ion at *m/z* 330.1830, indicating a molecular formula of $C_{20}H_{26}O_4$. The FTIR spectrum showed absorption bands at 3436 cm^{-1} for an OH stretch, 1731cm^{-1} for a carbonyl stretch and 1246 cm^{-1} for a C-O stretch. The ^1H NMR spectrum displayed three coupled broad singlet proton resonances at δ_H 6.42 (H-14), δ_H 7.45 (H-16) and δ_H 7.41 (H-15), indicative of a β-substituted furan ring. An oxymethine proton resonance at δ_H 5.47 (dd, *J*=5.0, 12.0), which corresponded to the carbon resonance at δ_C 72.4, was assigned as H-12 as it showed correlations in the HMBC spectrum with the C-13 (δ_C 125.9), C-14 (δ_C 108.8) and C-16 (139.7) resonances. The H-12 resonance also showed correlations with a lactone carbonyl carbon resonance (δ_C 172.8) assigned as C-17, and the C-10 resonance (δ_C 132.1). The C-17 resonance showed correlations with the H-8 (δ_H 2.31) and two H-7 resonances, and correlations were seen between the C-10 and 3H-20 (δ_H 1.19) and H-8 resonances. The H-3 oxymethine proton resonance at (δ_H 3.46) showed correlations with the C-18 (δ_C 20.4) and C-19 (24.5) methyl carbon resonances, C-1 (δ_C 24.3) and C-2 (δ_C 27.3) methylene carbon resonances and C-4 and C-5 carbon resonances (δ_C 39.8 and δ_C 135.1. The placement of the double bond between C-5 and C-10 (δ_C 132.1) was confirmed by correlations between the C-10 and the 2H-1 (δ_H 2.08), one of the H-2 (δ_H 1.81), 2H-6 (δ_H 2.11 and 2.22) and 2H-11 (δ_H 1.82 and 2.32) resonances and between the C-5 and the 2H-1, H-3, H-6 and H-7 proton resonances The coupling constants of the H-3 resonance (δ_H 3.46, dd, *J*=3.3, 11.7 Hz) indicated the α-orientation of H-3 (De, 1987; Graikou et al., 2005; Jones et al, 2007; Pudhom and Sommit, 2011). Correlations were seen between the H-3/ 3H-19, 3H-19/ 3H-20, 3H-20/H-12 resonances and therefore, these were confirmed to be on the α-face. The specific rotation for compound **5** was found to be +56.4, indicating the compound belonged to the *ent*-series (Hara et al., 1995).

The HREIMS data for compound **6** gave a molecular formula of $C_{20}H_{30}O_2$ for the compound ($[M + Na]^+$ at *m/z* 325.2138). The FTIR spectrum showed absorption bands at 3424 cm^{-1} for an OH stretch. Three coupled proton resonances at δ_H 6.28 (H-14), δ_H 7.35 (H-

15) and at δ_H 7.22 (H-16) showed that a β-substituted furan ring was present. These resonances showed coupling in the HMBC spectrum with a methylene C-12 carbon resonance (δ_C 18.5), and this, the absence of a lactone carbonyl carbon resonance, and the fact that four methyl groups were present in compound **6** as opposed to 3 in compound **5**, indicated that the 17,12-lactone was not present. The corresponding H-12 resonances (δ_H 2.36, 2H) were seen to be coupled to the two H-11 resonances, which were not further coupled, confirming the halimane structure. Coupling was seen in the COSY spectrum between the 3H-17 (δ_H 0.88, d, *J*=6.7)/H-8, H-8/2H-7 (δ_H 1.85, 1.90) and H-7/H-6 (δ_H 5.56) resonances. Thus a double bond was placed at C-5 (δ_C 142.5, C-5; δ_C 121.0, C-6). The C-5 resonance showed correlations in the HMBC spectrum with the H-3 (δ_H 3.49), 3H-18 (δ_H 1.07), 3H-19 (δ_H 1.15) and H-10 (δ_H 2.31) resonances. The C-10 resonance (δ_C 39.0) showed correlations with the 3H-20 (δ_H 0.71) and H-6 resonances.

The H-3 resonance occurred as a broad singlet (δ_H 3.49, brs $W_{1/2}$=8.6), indicating a β-equatorial orientation (Graikou et al., 2005; Liu et al., 2004), supported by correlations seen in the NOESY spectrum between the H-3 and both the H-2 resonances (δ_H 1.72 and 1.89) (Graikou et al., 2005). The H-3 proton resonance also showed correlations in the NOESY spectrum with the 3H-18 and 3H-19 resonances. Correlations seen between the H-8 (δ_H 1.62)/H-10 (δ_H 2.31) and H-10/3H-18 (δ_H 1.07) resonances confirmed that they were on the same face of the molecule.

Compound **19**, isolated as a yellow oily material, was found to be the C-10 epimer of the known maninsigin D which was isolated previously from *Manglietia insignis* Rehd (Magnoliaceae) (Shang et al., 2013). The HREIMS data showed a $[M-H]^+$ peak at *m/z* 233.1550, indicating a molecular formula of $C_{15}H_{22}O_2$ for the compound. The FTIR spectrum showed an OH stretch band at 3485 cm^{-1}. The ^{13}C NMR spectrum showed six aromatic carbon resonances at δ_C 120.9 (C-2), δ_C 122.5 (C-5), δ_C 128.2 (C-3), δ_C 137.5 (C-4), δ_C 137.6 (C-1) and δ_C 138.9 (C-6) for one benzene ring, two oxygenated fully substituted carbon resonances at δ_C 75.9 (C-10) and δ_C 80.2 (C-7), and two aliphatic methylene group carbon resonances at δ_C 24.4 (C-8) and δ_C 30.5 (C-9), four methyl group carbon resonances and one methine group carbon resonance. The ^1H NMR spectrum showed the presence of an isopropyl group at C-7 as in the co-isolated cadalene (**17**) and 4α,5β-corocalanediol (**18**) with two doublet methyl group proton resonances at δ_H 1.15 (d, *J*= 6.9 Hz, 3H-12) and δ_H 1.21 (d, *J* = 6.9 Hz, 3H-13) and one methine proton resonance at δ_H 2.60 (hept, *J* = 6.9 Hz, H-11).

The H-11 resonance showed correlations in the HMBC spectrum with the C-6, C-7 and C-8 resonances. A singlet methyl group proton resonance at δ_H 1.64 (3H-14) displayed correlations with the C-1, C-10, and C-9 (δ_C 30.5) resonances, confirming its assignment. The COSY spectrum showed coupling between the H-2 (δ_H 7.21, d, *J*= 8.0 Hz) and H-3 δ_H (7.18, d, *J*= 8.0 Hz) resonances and between the two H-8 (δ_H 2.36, δ_H 1.49) and the two H-9 (δ_H 1.59 and δ_H 2.26) methylene proton resonances. Thus the structure of this compound was shown to be 1,3,5-cadinatriene-7,10-diol. Four possible diastereomers are possible, one of which, 1,3,5-cadinatriene-7β,10β-diol (maninsigin D), has been reported (Shang et al., 2013). In order to confirm the structure, a ^{13}C NMR spectrum was run in CD_3OD to compare with the literature data of maninsigin D. Comparison of ^{13}C NMR chemical shifts of spectra run in CD_3OD showed differences especially at C-10, C-7 and C-14 which occurred at δ_C 81.6, δ_C 77.3 and δ_C 19.5 for compound **19**, and δ_C 73.5, δ_C 70.1 and δ_C 28.8 respectively for maninsigin D. Shang *et al*. used ROESY experiments to assign the relative configuration of maninsigin D, and concluded that the 3H-14 and the isopropyl groups were *cis* to each other. The NOESY spectrum of **19** showed no correlations between the 3H-14 resonance and the isopropyl group proton resonances (H-11, 3H-12 and 3H-13) as seen for manginsigin D, and this, in conjunction with the difference in ^{13}C NMR chemical shifts, suggested that the 3H-14 and the isopropyl group were *trans* to each other. Therefore, the absolute stereo structure for compound **19** would be either (7*R*,10*S*) (**19A**) or (7*S*,10*R*) (**19B**). The specific rotation for compound **19** was -96.0, compared to -9.4 reported for maninsigin D.

The absolute configuration of compound **19** was determined using electronic circular dichroism (ECD) studies. The experimental ECD curve showed negative Cotton effects at 201 nm (–18.68) and 307 nm (- 4.12) for compound **19**. The experimental ECD data was compared with calculated ECD curves for 3,5-cadinatriene-(7*R*,10*S*)-diol (**19A**) and 1,3,5-cadinatriene-(7*S*,10*R*)-diol (**19B**). A systematic analysis of conformer distribution using the molecular mechanics force fields (MMFF) at ground state was undertaken using Spartan14 software. MMFF analysis of 3,5-cadinatriene-(7*R*,10*S*)-diol (**19A**) gave 25 conformers, 21 of which were under 3 kcal/mol. The M0001 (-4.01 kcal/mol) conformer, which was consistent with the NOESY NMR experimental data and had a Boltzmann distribution of 0.775, was subjected to TDDFT calculations using a B3LYP method at 6-311G level built into Gaussian09 software. MMFF analysis of 1,3,5-cadinatriene-(7*S*,10*R*)-diol (**19B**) gave 24 conformers, 21 of which were under 3 kcal/mol. The M0001 (-4.01 kcal/mol) conformer, which had a Boltzmann distribution of 0.777, was subjected to TDDFT calculations using the

B3LYP method at 6-311G level built into Gaussian09 software. The calculated ECD curves for both diastereomers were compared to the experimental ECD curve for compound **19.** The calculated ECD curve of **19B** was found to be opposite and equal in intensity to the experimental curve but the calculated ECD curve for **19A** was similar, (Figure 2) confirming the structure as 1,3,5-cadinatriene-(7*R*,10*S*)-diol.

Compounds **2**, **5**, **6**, **7**, **8**, **11**, **12**, **14**, **17**, **18**, **19**, **20** were screened in vitro for cell viability against Caco-2 (human colorectal adenocarcinoma) cell lines using the neutral red assay (Repetto et al., 2008). The samples were tested at 100 µM to estimate their cytotoxicity and compared with actinomycyin at a concentration of 7.96 µM (10µg/ml) as a positive control as described in the Experimental Section. Only compound **19** reduced Caco-2 at 100 µM (Figure S4 in Supporting Information) and was further tested at five concentrations, 1, 3, 10, 30 and 100 µM. Compound **19** reduced cell viability at 10 µM (Figure 3). None of the compounds isolated have been screened previously for cytotoxicity although maninsigin D has been tested against HL-60, A-549, SW-480, SMMC-7721 and MCF-7 human cell lines by the MTT method and showed IC_{50} values > 40 µM (Shang et al., 2013) and the normal enantiomer of **12** showed cytotoxicity against BT-474, KATO-3, CHAGO, SW-620 and HEP-G2 human cancer cell lines with LC_{50} values of 3.26, 6.78, 6.67, 4.44 and 6.37 µg/mL (Pudhom and Sommit, 2011).

Compounds **2**, **3**, **5**, **6**, **11**, **12**, **19** and **20** were evaluated against the NCI59 developmental therapeutics program 59 cancer cell line screen at one dose of $1x10^{-5}$M (NCI DTP Developmental Therapeutics Program, 2016). The compounds did not meet the criteria for further testing (Supporting Information).

The compounds isolated in this work are consistent with our previous investigations of African *Croton* species, which have been shown to yield a wide range of diterpenoid classes as the major constituents, along with sesquiterpenoids and common triterpenoids (Mulholland et al., 2010; Langat et al., 2011, 2012; Ndunda et al., 2016).

3. Experimental section

3.1 General Experimental Procedures

Optical rotations were measured at room temperature on a JASCO-P-1020 polarimeter and IR spectra were recorded using a Perkin-Elmer (2000 FTIR) spectrophotometer using KBr discs. ECD spectra were measured on a Chirsascan CD spectrometer. ^{1}H, ^{13}C and 2D

NMR spectra were recorded on a Bruker AVANCE III NMR spectrometer, operating at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C, using standard experiments from the Bruker pulse programs library. Chemical shifts are reported in ppm (δ) referencing the solvent signal ($CDCl_3$) as internal standard respect to TMS (0 ppm), and coupling constants (*J*) are measured in Hz. HREIMS was performed on a Bruker MicroToF Mass Spectrometer, using an Agilent 1100 HPLC to introduce samples.

3.2 Plant Material

The root of *Croton dichogamus* Pax was collected in Wamunya, in the Machakos area of Kenya (1.41°S, 37.62°E) and identified by Mr. Patrick Mutiso of the University Herbarium, School of Biological Studies, University of Nairobi, Kenya. A voucher specimen (BN 2010/13) was deposited at the Herbarium, School of Biological Studies, University of Nairobi.

3.3. Extraction and Isolation

The ground root (1 kg) of *Croton dichogamus* Pax was extracted at room temperature on a shaker for 24 h successively with *n*-hexane, CH_2Cl_2 and MeOH, to yield, after evaporation of the solvents, *n*-hexane (8.5 g), CH_2Cl_2 (20.0 g) and MeOH (25.6 g) extracts. The crude extracts were separated using medium pressure flash chromatography (Grace Reveleris X2) over silica gel using a *n*-hexane/CH_2Cl_2/ MeOH step gradient and collecting 20 mL fractions. The compounds were detected using an ELSD detector at three wavelengths, 320, 280 and 254 nm. Subsequent purifications were carried out using preparative thin layer chromatography (silica gel 60 F254 coated aluminium backed TLC sheets) and/or 1 cm diameter column, which was packed with Sephadex (LH 20) in MeOH/ CH_2Cl_2 or silica gel (Merck Art. 9385). Detailed extraction procedures are provided as Supporting Information (Schemes S1.1, S1.2 and S1.3). The *n*-hexane extract contained cadalene **17** (3.9 mg), 6α-methoxy-patchoulan-4-ene (6α-methoxycyperene) **16** (7.7 mg), depressin **4** (6.0 mg), 3β,4β,15,16-diepoxy-13(16),14-clerodadiene **13** (4.3 mg), crotodichogamoin A **2** (6.2 mg), 1,3,5-cadinatriene-(7*R*,10*S*)-diol **19** (3.1 mg), crotodichogamoin B **3** (8.0 mg), 3β,4β,15,16-diepoxy-13(16),14-*ent*-clerodadiene-17,(12*S*)-olide **10** (4.2 mg), acetyl aleuritolic acid **20** (13.5 mg), 15,16-epoxy-3-hydroxy-5(10),13(16),14-*ent*-halimatriene-17,(12*S*)-olide **5** (2.8 mg) and 4-patchoulen-3-one **15** (3.2 mg). The CH_2Cl_2 extract gave 4-patchoulene **14** (5.2 mg), 4-patchoulen-3-one **15** (1.7 mg), cadalene **17** (7.1 mg), 1,3,5-cadinatriene-(7*R*,10*S*)-diol

19 (1.5 mg), 15,16-epoxy-13(16),14-*ent*-clerodadien-3-one **12** (7.5 mg), crotodichogamoin A **2** (3 mg), 15,16-epoxy-5,13(16),14-*ent*-halimatriene-3-ol **6** (1.8 mg), 15,16-epoxy-4(18),13(16),14-*ent*-clerodatrien-3α-ol **11** (1.6 mg), acetyl aleuritolic acid **20** (3.3 mg), crotodichogamoin B **3** (2.9 mg), 15,16-epoxy-3-keto-3(16),14-*ent*-clerodadien-17,(12*S*)-olide **8** (10.2 mg), 15,16-epoxy-3β-hydroxy-*ent*-halimatriene-17,(12*S*)-olide **5** (3.2 mg), 1(6),7,9-cadinatriene-4α,5β-diol **18** (3.0 mg) and 15,16-epoxy-3α,4β-dihydroxy-3(16),14-*ent*-clerodadien-17,12*S*-olide **7** (15.4 mg). The MeOH extract gave 15,16-epoxy-3α-hydroxy-4(18),13(16),14-*ent*-clerodatrien-17,(12*S*)-olide **9** (4.2 mg**)**, 3β,4β,15,16-diepoxy-13(16),14-clerodadiene **13** (4.7 mg) and crotohaumanoxide **1** (2.7 mg).

*3.3.1. Crotodichogamoin A (**2**)*

Yellow gum; $[\alpha]^{23}_{D}$ + 11.3 (c 0.020, $CHCl_3$); IR (KBr) ν_{max} 2917, 2848, 1747, 1649 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 327.1590 $[M + H]^+$ (calcd for $C_{20}H_{22}O_4$+H, *m/z* 327.1597).

*3.3.2. Crotodichogamoin B (**3**)*

Yellow oil; $[\alpha]^{23}_{D}$ + 111.1 (c 0.001, $CHCl_3$); IR (KBr) ν_{max} 2928, 2856, 1730, 1594 cm^{-1}; UV (ACN); λ_{max} = 306, 242, 219 nm; ^{1}H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 295.16945 $[M + H]^+$ (calcd for $C_{20}H_{22}O_2$+H, *m/z* 295.1699).

*3.3.3. 15,16-Epoxy-3β-hydroxy-5(10),13(16),14-ent-halimatriene-17,(12S)-olide (**5**)*

White solid; $[\alpha]^{23}_{D}$ + 56.0 (c 0.036, MeOH); IR (KBr) ν_{max} 3436, 2936, 2876, 1731 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HREIMS *m/z* 330.1830 $[M]^+$ (calcd for $C_{20}H_{26}O_4$, *m/z* 330.1831).

*3.3.4. 15,16-Epoxy-5,13(16),14-ent-halimatriene-3-ol (**6**):*

Yellow oil; $[\alpha]^{23}_{D+}$ 40.5 (c 0.032, MeOH); IR (KBr) ν_{max} 3424, 2922, 2852 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HREIMS *m/z* 325.2138 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_2$+Na, *m/z* 325.2144).

*3.3.5 1,3,5-Cadinatriene-(7R,10S)-diol (10-epi-maninsigin D) (**19**)*

Yellow oil, $[\alpha]^{23}_{D}$ -96.0 (c 0.002,MeOH); IR (KBr) ν_{max} 3485, 2926, 2857, 1601 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HREIMS *m/z* 233.1550 $[M-H]^{+}$ (calcd for $C_{15}H_{22}O_2$-H, *m/z* 233.1541).

3.4. Cytotoxicity Assay

Caco-2 human colon carcinoma cells (CACO-2 (ECACC 86010202) from Public Health England, UK) were cultured in Eagles' Modified Essential Medium supplemented with 10% Foetal Bovine Serum, 2 mM glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and maintained in 5% CO_2/95% air in an incubator at 37°C. Cells were plated in 96 well plates at $2x10^4$ cells/well and incubated for 48 hours. Cells were then treated for 48 hours with one of the following, in 4 replicates: culture medium alone (untreated control); 0.1% DMSO (vehicle control; maximum DMSO exposed to cells); actinomycin D 10 µg/ml (positive control); test substances at 1, 3, 10, 30 and 100 µM (diluted from a 100 mM stock in DMSO). Cells were washed with PBS, and neutral red (25 µg/ml in culture medium, diluted from a stock of 2.5 mg/ml in ultrapure water) was added for 3 hours, cells were washed with PBS, and neutral red extracted in 50% ethanol/water with 1% acetic acid. Absorbance was read at 540 nm. Data are from 2-3 independent experiments (different cell passages). The mean absorbance of wells without cells (blank) was subtracted from all other readings. Replicates were averaged to give one treatment value per plate. All plate treatment values were divided by the mean value of vehicle-treated cells on the same plate, so data was normalised as percentage of maximum cell viability.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at xxxxx

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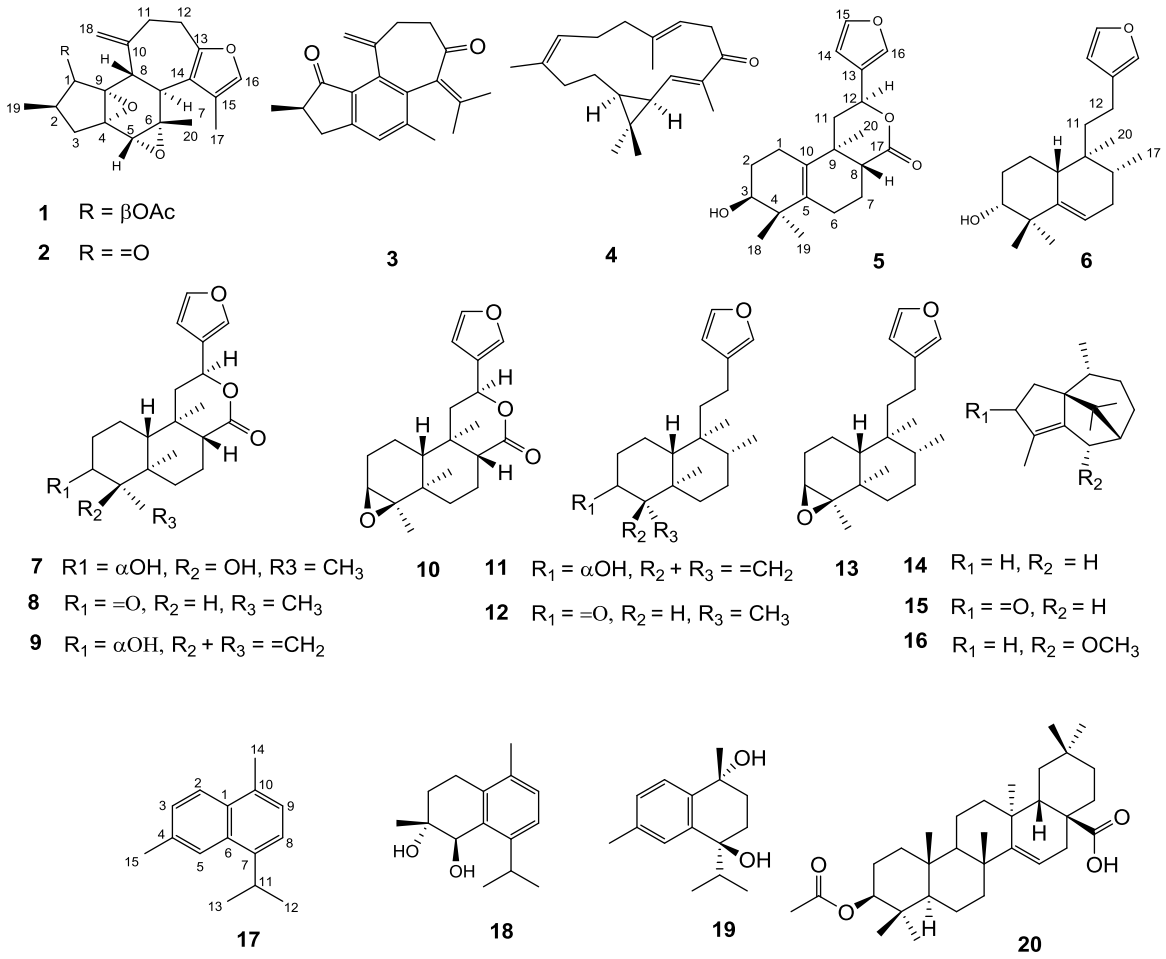

Figure 1. Compounds isolated from the roots of *Croton dichogamus*

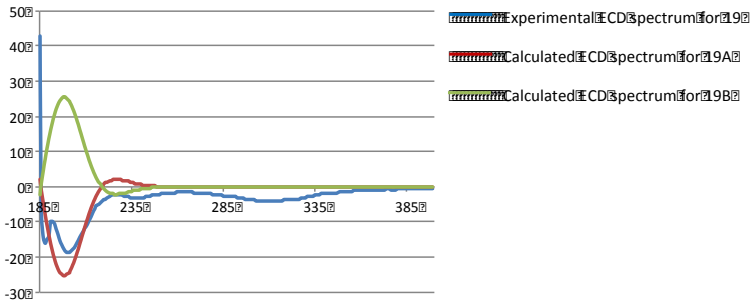

Figure 2: Experimental ECD spectrum for compound 19 (blue) and TDDFT simulated spectra for 19a (red) and 19b (green). (Units for the y-axis are (Δε) and λ (nm) for the x-axis.)

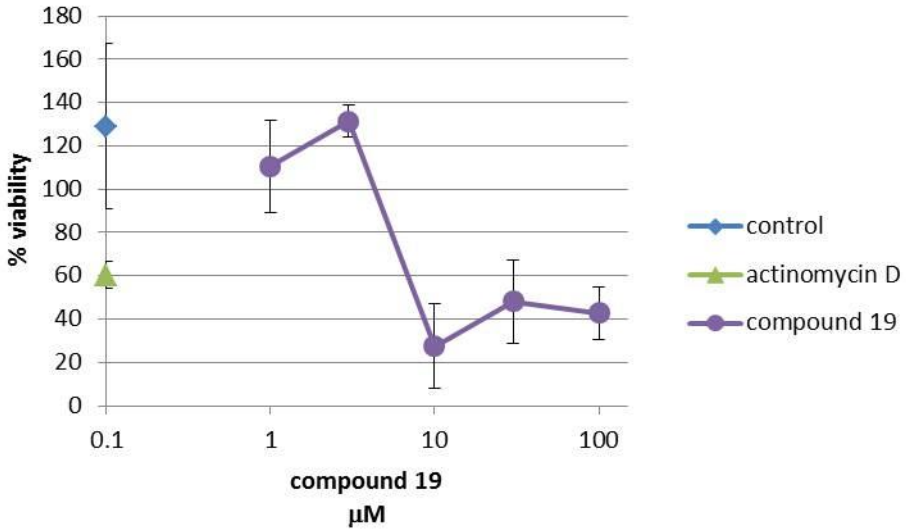

Figure 3: Effect of compound 19 on Caco-2 cell viability after 48 hours. Viability relative to vehicle (0.1% DMSO)-treated cells is shown. Mean values with standard deviation are plotted from N=2 (1- 30 µM) or N = 3 (100 µM) experiments. Actinomycin D (10 µg/ml) was used as the positive cytotoxic control.

Table 1. NMR spectroscopic Data (500 MHz, $CDCl_3$) of compounds 1-3

	1			2			3		
position	$\delta_{C,\ type}$	δ_H (*J* in Hz)	HMBC	δ_C, type	δ_H (*J* in Hz)	HMBC	δ_C, type	δ_H (*J* in Hz)	HMBC
1	75.3, CH	5.50, d (5.6)	2, 3, 4, 8, 9	206.1, C			208.0, C		
2	33.8, CH	2.21, m	3, 19	38.7, CH	2.49, m[a]	1, 3, 4, 9, 19	42.9, CH	2.72, m[a]	1, 3, 9, 19
3a	37.2, CH_2	2.47, dd[a] (13.7, 7.3)	1, 2, 4, 9	31.2, CH_2	2.66, dd (14.0, 8.3)	1, 2, 4, 9, 19	34.4, CH_2	3.34, dd (16.8, 8.0)	1, 2, 4, 5, 9, 19
3b		1.64, dd (13.7, 7.3)	1, 2, 4, 5, 19		1.84 dd (14.0, 6.8)	1, 2, 4, 9, 19		2.66, m	2, 4, 5, 9,19
4	60.7, C			65.8, C			131.0, C		
5	57.8, CH	3.14, s	3, 4, 6, 9, 20	56.2, CH	3.29, s	4, 6, 9, 20	126.9, CH	7.22, s	3, 4, 6, 7, 9, 20
6	57.4, C			61.5, C			137.9, C		
7	41.8, CH	2.80, d (12.5)	5, 6, 8, 9, 10, 13, 14, 15, 20	49.0, CH	2.49, d[a] (13.0)	6, 8, 9, 10, 13, 14, 15, 20	143.4, C		
8	37.1, CH	3.06, d (12.5)	6, 7, 9, 10, 11, 18	39.9, CH	2.78, d[a] (13.0)	4, 6, 7, 8, 9, 10, 14, 18	139.2, C		
9	69.0, C			66.1, C			153.3, C		
10	145.3, C			141.9, C			142.0, C		
11a	36.5, CH_2	2.58, m	8, 10, 12, 18	37.8, CH_2	2.53, m	8, 10, 12, 13, 18	31.7, CH_2	2.82, m	8, 10, 12, 13, 18
11b		2.47, m[a]	8, 10, 12, 13, 18		2.78, m[a]	10, 12, 13, 18		2.72, m[a]	8, 10, 12, 13, 18
12a	22.9, CH_2	2.98, m	11, 13, 14	23.1, CH_2	2.81, m	10, 11, 14	40.9, CH_2	2.27, m	10, 11, 13, 14
12b		2.77, m	10, 11, 13		2.95, m	10, 11, 13, 14		2.43, m	10, 11, 13
13	150.4, C			150.4, C			202.5, C		
14	122.3, C			121.8, C			134.3, C		
15	117.5, C			117.0, C			147.5, C		
16	137.0, CH	7.0, brs (3.49)	13, 14, 15	137.0, CH	7.03, s	13, 14, 15, 17	23.5, CH_3	1.49, s	14, 15, 17
17	8.8, CH_3	1.96, brs (2.53)	14, 15, 16	8.9, CH_3	1.96, s	14, 15, 16	22.1, CH_3	2.13, s	14, 15, 16
18	114.1, CH_2	4.91, brs (4.93)	8, 10, 11	116.9, CH_2	5.06, s	8, 10, 11	114.8, CH_2	5.26, s	
18		5.01, brs (4.85)	8, 10, 11		4.67, brs	8, 10, 11		4.93, brs	
19	12.8, CH_3	0.91, d (7.1)	1, 2, 3	16.0, CH_3	1.11, d (7.5)	1, 2, 3	16.1, CH_3	1.28, d (7.1)	1, 2, 3
20	20.2, CH_3	1.08, s	5, 6, 7	20.3, CH_3	1.09, s	5, 6, 7	20.6, CH_3	2.21, s	8, 10, 11
COO	169.7, C								
$COCH_3$	20.8, CH_3	2.11, s	1, COO						

[a]Overlapping values

Table 2. NMR spectroscopic Data (500 MHz, $CDCl_3$) of compounds 5, 6, 11 and 19

	5		**6**		**11**		**19**	
position	$\delta_{C,\ type}$	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	24.3, CH_2	2.08, m	37.1, CH_2	1.55, m[a] 1.68, m	16.9, CH_2	1.83, dd (3.2, 12.8) 1.47, m[a]	137.6, C	
2α 2β	27.3, CH_2	1.66, m 1.81, m	28.5, CH_2	1.72, m 1.89, m	34.9, CH_2	2.03, m 1.57, m	120.9, CH	7.21, d[a] (8.0)
3	75.9, CH	3.46, dd (11.7, 3.3)	76.6, CH	3.49, brs ($W_{1/2}$ 8.6)	74.9, CH	4.34, brs ($W_{1/2}$ 7.8)	128.2, CH	7.18, d (8.0)
4	39.8, C		41.5, C		161.0, C		137.5, C	
5	135.1, C		142.5, C		40.1, C		122.5, CH	7.20, s[a]
6α	25.0, CH_2	2.11, m	121.0, CH	5.56, brd (10.8)	38.1, CH_2	1.72, m	138.9, C	
6β		2.22, m				1.47, m[a]		
7α 7β	18.6, CH_2	1.60, m 2.30, m[a]	32.0, CH_2	1.85, m 1.90, m	27.3, CH_2	1.46, m[a] 1.52, m	80.2, C	2.36, ddd (13.0, 9.6, 3.5) 1.49, dt (12.4, 3.9,)
8a 8b	48.3, CH	2.31, m[a]	33.7, CH	1.62, m	36.8, CH		24.4, CH_2	2.36, ddd (13.0, 9.6, 3.5) 1.49, dt (12.4, 3.9)
9a 9b	37.0, C		37.4, C		39.5, C	1.17, dd (1.9, 12.1)	30.5, CH_2	1.59, dt (12.4, 3.9) 2.26, ddd (13.0, 9.6, 3.5)
10	132.1, C		39.0, CH	2.31, m	48.9, CH	1.63, m	75.9, C	
11α	41.1, CH_2	2.32, m	20.3, CH_2	1.56, m[a]	38.8, CH_2	1.51, m	30.6, CH	2.60, hep (6.9)
11β		1.82, m				2.29, td (4.5, 13.5)		
12	72.4, CH	5.47, dd (12.0, 5.0)	18.5, CH_2	2.36, m	18.5, CH_2	2.13, td (4.5, 13.5)	17.1, CH_3	1.15, d (6.9)
13	125.9, C		125.9, C		125.9, C		18.7, CH_3	1.21, d (6.9)
14	108.8, CH	6.42, brs ($W_{1/2}$ 4.0)	111.2, CH	6.28, brs ($W_{1/2}$ 3.9)	111.2, CH	6.24, brs ($W_{1/2}$ 3.9)	19.3, CH_3	1.64, s
15	143.9, CH	7.41, brs ($W_{1/2}$ 4.1)	143.0, CH	7.35, brs ($W_{1/2}$ 4.0)	142.9, CH	7.34, brs ($W_{1/2}$ 4.0)	21.9, CH_3	2.41, s
16	139.7, CH	7.45, brs ($W_{1/2}$ 3.7)	138.6, CH	7.22, brs ($W_{1/2}$ 3.8)	138.6, CH	7.19, brs ($W_{1/2}$ 3.8)		
17	172.8, C		15.3, CH_3	0.88, d (6.7)	16.3, CH_3	0.83, d ($W_{1/2}$ 6.0)		
18	20.4, CH_3	0.98, s	28.6, CH_3	1.07, s	109.7, CH_2	4.87, brs ($W_{1/2}$ 3.0) 4.81, brs ($W_{1/2}$ 3.2)		
19	24.5, CH_3	1.07, s	25.7, CH_3	1.15, s	23.2, CH_3	1.26, s		
20	20.3, CH_3	1.19, s	16.4, CH_3	0.71, s	18.4, CH_3	0.79, s		

[a]Overlapping values