Cembranolides from the stem bark of the southern African medicinal plant, *Croton gratissimus* (Euphorbiaceae)

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Abstract: The stem bark of *Croton gratissimus* (Euphorbiaceae) yielded four novel cembranolides, including the first reported example of a 2,12-cyclocembranolide, (+)–[1*R**,2*S**,7*S**,8*S**,12*R**]–7,8-epoxy-2,12-cyclocembra-3*E*,10*Z*-dien-20,10-olide, whose structure was confirmed by means of single crystal X-ray analysis. This compound showed moderate activity against the PEO1 and PEO1TaxR ovarian cancer cell lines.

Keywords: *Croton gratissimus*, Euphorbiaceae, cembranoids, (+)–[1*R**,2*S**,7*S**,8*S**,12*R**]–7,8-epoxy-2,12-cyclocembra-3*E*,10*Z*-dien-20,10-olide; (+)–[1*R**,10**R*]-cembra-2*E*,4*E*,7*E*,11*Z*-tetraen-20,10-olide; (+)–[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide; (-)–[1*R**,4*R**,10*R**]-4-hydroxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide; Logic for Structure Determination Programme; PEO1 and PEO1TaxR ovarian cancer cell lines; Single crystal X-ray analysis.

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1. Introduction:

Of the approximately 750 species of *Croton* L. (Euphorbiaceae) distributed throughout the tropics, some 50 are found in Africa (Mabberley 2008), and only 10 species are native to the *Flora of southern Africa* region. *Croton gratissimus* Burch. (syn. *C. zambesicus* Müll. Arg.; *C. microbotryus* Pax.) is represented by two varieties, the typical one (*C. gratissimus* Burch. var. *gratissimus*) being the subject of this account. This semi-deciduous tree species is widespread in sub-Saharan Africa, occurring on stony or rocky hillsides throughout much of the warmer and drier regions, from South Africa northeastwards to the horn of Africa. The leaves produce a pleasant lavender-like scent when crushed, and are used dried and powdered for their perfume (Palmer and Pitman, 1972). Across its range it is an important ethnomedicinal species: the Zulu use milk infusions of the bark as purgatives for stomach and intestinal disorders, despite its toxic reputation (Bryant, 1966). Elsewhere several other crotons including the Asian *C. tiglium* L., and *C. flavens* L. from the Caribbean have been so employed, although diterpenes from both have been implicated in indirect carcinogenesis (oesophageal cancer) through activation of the Epstein-Barr virus (Hecker, 1981; Bruneton, 1995). The Zulu further treat unspecified uterine disorders with powdered bark blown into the womb. They also remedy pleurisy or pleurodynia by rubbing the powdered bark into chest skin incisions to act as a counter-irritant, given its cutaneous eruptive irritant and stimulant properties (Bryant 1966). As a cure for insomnia and restlessness, the leaves are ground with goat fat and those of two other *Croton* species, the paste heated on coals and the fumes inhaled (Palmer and Pitman, 1972). Gerstner (1941) further recorded the purgative properties of the roots, and documented their application in treating fevers. Zimbabweans treat coughs with smoke from leaves, and take root infusions for abdominal pains and as an aphrodisiac (Gelfand *et al*., 1985). In Botswana a decoction prepared with leaves is taken for coughs (Hedberg and Staugård, 1989). Watt and Breyer-Brandwijk (1962) documented the use of *C. gratissimus* bark in treating painful respiratory conditions (including intercostal neuralgia), unspecified plant parts as a remedy for fevers, charred, powdered bark for bleeding gums, and leaves to treat both eye disorders and rheumatism. In Venda, leaves are dried and smoked for influenza, colds and fevers (Mabogo, 1990). Doubts about the toxicity of this species have been raised due to the esteem with which leaves have been held as a stock food in Namibia (Watt and Breyer-Brandwijk, 1962).

In this country roots and leaves of this taxon have found application as a treatment for colds and coughs, bark for ear problems, and roots for chest ailments and fever (Von Koenen, 2001). From the above usage profiles of *C. gratissimus* the following diverse bioactivities are indicated: analgesia, febrifugal, aphrodisiac, purgative, emetic, soporific, antibiotic and antiviral. The febrifugal activity of *C. gratissimus* (in the context of malaria) has earlier been demonstrated: Clarkson *et al*. (2004) found DCM extracts of the leaves to show a high antiplasmodial activity *in vitro* of 3.5 µg/ml. In the current study, congnisance has been taken of the use of this species for unspecified abdominal pains (Gelfand *et al*., 1985), an indication of potential antineoplastic applications (Charlson, 1980), especially as the Zulu treat unspecified uterine disorders with powdered bark preparations (Bryant, 1966). Accordingly, isolates from the bark were screened against PEO1 and PEO1TaxR ovarian cancer cell lines.

Previous investigations of the genus *Croton* have yielded pimarane (Block *et al.*, 2004), kaurane (Kuo *et al*., 2007), labdane (Garcia *et al*., 2006), clerodane (Garcia *et al*., 2006) and cembrane (Pudhom *et al*., 2007) diterpenoids, isoquinoline alkaloids (New World species only) (Charris *et al*., 2000) and triterpenoids (Block *et al*., 2004).

2. Results and discussion:

The hexane and dichloromethane extracts of the stem bark of *Croton gratissimus* yielded lupeol, 4(15)-eudesmene-1β,6α-diol, α-glutinol and four novel cembrane diterpenoids, **1 - 4**.

HREIMS of compound **1** indicated a molecular formula of $C_{20}H_{28}O_3$. The IR spectrum showed a peak at 1777 cm^{-1} and a resonance at δ183.6 indicating the presence of a carbonyl group. Interestingly, this was the only one of the four compounds that was very stable on standing and crystalline. These spectroscopic values differed considerably from the carbonyl stretch which occurred at 1733-1740 cm^{-1} in the IR spectra and at approximately δ174 in the ^{13}C NMR spectra of compounds **2-4**. The ^{13}C NMR spectrum showed that compound **1** had two trisubstituted double bonds (δ128.5 (CH), δ136.0 (C); δ151.2 (C), δ110.6 (CH))

indicating, in conjunction with the molecular formula, that the molecule contained four rings. The ^{1}H NMR spectrum showed the presence of two secondary methyl groups (δ 0.85 (d, *J*=6.6 Hz), 0.92 (d, *J*=6.6 Hz)) which were ascribed to H_3-16 and H_3-17 of a cembranoid structure. These resonances were seen to be coupled to the H-15 resonance (δ1.68) which was coupled further to the H-1 resonance at δ1.70. The H-1 resonance showed further coupling with the two H-14 resonances (δ1.84 and δ1.67), and a single proton resonance which could be ascribed to H-2 (δ 2.91, t, *J* = 9.6 Hz). The H-2 resonance showed coupling in the COSY spectrum with an alkene proton resonance (δ 4.85, d, *J*=9.6 Hz) which was assigned to H-3, enabling placement of the first double bond at C-3. The H-3 resonance showed long range coupling with the H_3-18 proton resonance at δ1.52. The corresponding C-18 resonance occurred at δ15.1, confirming the *E*-configuration of the double bond (Lange *et al.*, 1986). Interestingly, the H-2 resonance showed correlations in the HMBC spectrum with the carbonyl carbon resonance (δ183.6) and the second alkene carbon resonance (δ110.6), suggesting a linkage across the cembranoid. The presence of an epoxide ring was suggested with resonances at δ63.0 (CH) and δ 61.9 (C) leaving a methyl group, and four further methylene groups to be placed. Use was made of the Logic for Structure Determination Program to complete the structure of the molecule and to rule out the possibility of any other possible structure. According to the usual LSD protocol (Nuzillard, 2003; Nuzillard, 2008), the ^{13}C NMR resonances of **1** were first identified by numbers ranging from 1 to 20, in the decreasing order of chemical shift values. The ^{1}H NMR resonances were identified using the HSQC spectrum, so that a carbon atom and a hydrogen atom that are directly bonded share the same identifier. Then, a status was assigned to each atom, which is defined by its chemical element, its hybridization state and the number of bonded hydrogen atoms (also named, multiplicity). Only the carbon atoms whose chemical shift is over 110 ppm are doubtless sp^2 hybridized. There must be an even number of sp^2 atoms in a molecule and therefore one of the oxygen atoms is sp^2. Carbon 1 (LSD numbering) at δ 183.62 is thus supposed to be doubly bonded to an oxygen atom. The number of hydrogen atoms that are bonded to each carbon atom was determined from the 1D *J*-modulated ^{13}C NMR spectrum and from the 2D HSQC spectrum. Overall, 28 hydrogen atoms were counted, leading to the presence of 2 sp^3 oxygen atoms with no exchangeable proton. For each atom, the status and the

identifiers of the protons that correlate in the HMBC spectrum are reported in Supplementary Material 1. Moreover, the protons of the methyl groups 17 and 19 appear as doublets and therefore carbons 17 and 19 have a methine group as neighbours. In addition, the protons of the methyl groups 18 and 20 appear as singlets and therefore carbons 18 and 20 have quaternary carbons as neighbours. With these constraints, LSD produced 169 structures. All carbons with $\delta < 34$ were then considered to have only carbon atoms as neighbours. LSD then produced 13 solutions. In many of them, there were bonds between sp^3 oxygens and sp^3 carbons that were not really compatible with the measured ^{13}C NMR chemical shifts, unless an epoxide were present. Only three solutions were produced after an epoxide was imposed. The first one contained a cyclobutanone fragment for which the chemical shift of C-1 was not realistic. In the second one, C-10 (LSD numbering) was bonded to an oxygen atom within a five-membered lactone ring. The chemical shift of C-10 at δ 53.17 was not realistic. The third solution was the one that was finally retained for compound **1**. The unusual cyclic enol ester group correctly explained the strong chemical shift difference of C-10 and C-11 (structure **1** numbering). The LSD input file for this determination is supplied as Supplementary Information 2. The molecule is the first example of a cembrane-derived diterpenoid with a C-2, C-12 bond, resulting in a spirocyclic C-12.

The NOESY spectrum showed correlations between the H_3-16 and H_3-17 resonances and H-2, between H-2 and the H_3-18 resonance, and between the H_3-18 and H_3-19 resonances indicating they were on the same face of the molecule, and between H-1 and H-3, H-3 and H-11 and between H-11 and H-7, showing they were on the opposite face. Single crystal X-ray analysis (Fig 1) confirmed the structure and relative stereochemistry as (+)–[1*R**,2*S**,7*S**,8*S**,12*R**]–7,8-epoxy-2,12-cyclocembra-3*E*,10*Z*-dien-20,10-olide.

Compound **2** was isolated as a colourless oil and identified as (+)–[1*R**,10*R**]-cembra-2*E*,4*E*,7*E*,11*Z*-tetraen-20,10-olide. The HRMS indicated a molecular formula of $C_{20}H_{28}O_2$ and the FTIR spectrum gave an absorption band at 1745 cm^{-1}, consistent with an α,β–unsaturated γ-lactone (Pavia *et al*., 2009). The ^{13}C NMR spectrum displayed twenty carbon resonances, including a carbonyl carbon resonance at δ 174.0 and eight double bond carbon resonances. The ^{1}H NMR spectrum showed the

presence of four methyl group proton resonances at δ 1.70 (s), 1.78 (s) and 0.89 (d, *J* = 6.8 Hz) and 0.82 (d, *J* = 6.8 Hz), indicating two vinylic methyl groups (H_3-19, H_3-18, respectively), and the presence of an isopropyl group, as in **1**. The two methyl group doublets (H_3-16, H_3-17) were each seen to be coupled to a methine proton resonance at δ 1.55 (H-15), which showed further coupling with a resonance at δ 1.72 (m) (H-1). The H-1 resonance was seen to be coupled with an alkene proton resonance at δ 5.26 (dd, *J* =15.4, 10.0 Hz) (H-2), which showed further coupling with a doublet proton resonance at δ 6.00 (d, *J*=15.4 Hz), ascribed to H-3. The configuration of the Δ^2- double bond was assigned as *E*, based on the magnitude of the coupling constant $J_{2,3}$ = 15.4 Hz (Olsson *et al*., 1993). The HMBC spectrum showed correlations between the H-3 proton resonance and the C-1 (δ 50.1), C-2 (δ 130.2), C-4 (δ 135.3), C-5 (δ 125.1) and C-18 (δ 19.1) resonances. The ^{13}C NMR chemical shift for the C-18 methyl group of δ 19.6 indicated an *E* geometry for the Δ^4 - double bond, therefore the H_3-18 methyl group was *trans* to the H-5 (δ 5.49 t *J* = 7.5, 15.0 Hz) alkene proton (Crombie *et al*., 1975, Lange *et al*., 1986, Olsson *et al*., 1993, Pudhom *et al*., 2007). The H-5 resonance showed coupling with two H-6 proton resonances (δ 2.41 (m) and 3.05 (m)), which, in turn, showed coupling with the H-7 (δ 5.10, d, *J*=9.5 Hz) alkene proton resonance. The H-7 resonance showed correlations in the HMBC spectrum with the C-6 (δ 26.4), C-19 (δ 18.0) and C-9 resonances (δ 41.9). The chemical shift for C-19 (δ 18.0) indicated an *E* geometry for the Δ^7-double bond (Lange *et al*., 1986). The H_2-9 resonances (δ 2.78, d, *J* = 14.5 Hz and 2.46, m) were seen to be coupled with the H-10 resonance (δ 5.04, br s, $W_{1/2}$ = 8.1 Hz) which showed further coupling with H-11 of the α,β–unsaturated γ-lactone. The lactone carbonyl carbon showed correlations in the HMBC spectrum with the overlapped H_2-13 resonances (δ 2.33, m). The H_2-13 resonances showed coupling with the two H-14 resonances (δ 2.41 (m), 1.38 (m)), which showed coupling with the previously assigned H-1 resonance. The H_3-18 and H_3-19 methyl groups were placed on the β face of the molecule as for compound **1,** and, following compound **1,** where the stereochemistry at C-1 was determined to be *R,* the isopropyl group was placed in the β–orientation. Note that it is the conformation which the molecule adopts that results in such substituents, attached to sp2 carbons, pointing above or below the general plane of the molecule. The H-10 proton was determined to be in the β-configuration due to an observed correlation in the NOESY spectrum with the H_3-19 proton

resonance. The optical rotation for compound **2** was found to be +23.77. Compound **2** was identified as (+)–[1*R**,10*R**]-cembra-2*E*,4*E*,7*E*,11*Z*-tetraen-20,10-olide.

Compounds **3** and **4** were found to be C-4 epimers and HRMS analysis indicated a molecular formula of $C_{20}H_{30}O_3$. They differed from compound **2** in having a hydrated Δ^4-double bond. The IR spectrum of **3** showed a carbonyl stretch at 1738 cm^{-1}, indicating an α,β-unsaturated 5-membered lactone (Pavia *et al*., 2009) and a O-H stretch band at 3411 cm^{-1}. A tertiary hydroxyl group was present at C-4 (δ 72.5), and the H_3-18 methyl group proton resonance now occurred at δ 1.37.

The relative stereochemistry at the C-1, C-4 and C-10 chiral centres was assigned using the NOESY experiment and by comparisons with compound **1**. The stereochemistry at C-1 was arbitrarily assumed to be the same as for compound **1,** and H-1 was assigned the α-configuration. The H-1 proton resonance showed a correlation in the NOESY spectrum with the C-4 tertiary hydroxy group proton resonance at δ 4.36 (s) when the spectrum was acquired in DMSO-d_6. This hydroxy group was therefore placed in the α-orientation and hence the H_3-18 (δ1.37) methyl group proton was assigned as β. A correlation seen between the H_3-18 methyl group proton resonance and the isopropyl group (H-15 and H_3-16) in the NOESY spectrum confirmed this. The H_3-19 methyl group proton resonance (δ1.67, s) showed a correlation with the β–orientated H_3-18 resonance and the H-10 (δ5.03, br t, $W_{1/2}$ = 19.6 Hz) oxymethine proton resonance, hence H-10 was also determined to be β–configured. The ^{13}C NMR chemical shift of the C-19 methyl group of δ16.5 (< 20 ppm) (Lange *et al*., 1986) and the absence of a correlation between the H-7 (δ5.37, t, *J*=7.5 Hz) proton resonance and the H_3-19 (δ1.67, s) methyl group proton resonance in the NOESY spectrum indicated that the C-7 (δ131.1) , C-8 (δ129.4) double bond was in the *E* configuration. Compound **3** was identified as (+)–[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*, 7*E*,11*Z*-trien-20,10-olide.

Compound **4** was found to be (-)–[1*R**,4*R**,10*R**]-4-hydroxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide. The ^{13}C NMR chemical shifts for compound **4** were found to be very similar to those of compound **3**, except for the C-18 carbon resonance at δ 30.3 (δ28.2 for **3**) and C-4 resonance at δ73.6 (δ72.5 for **3**). The H-1 (δ1.58) proton

resonance was assigned as α, as in compound **1.** The presence of a correlation between the H-1 proton resonance and the H_3-18 methyl group proton resonance in the NOESY spectrum allowed for the assignment of the α-orientation of the H_3-18 methyl group. Based on the ^{13}C NMR chemical shift for C-19 (δ16.5 < 20 ppm) (Lange *et al*., 1986) the H_3-19 methyl was also placed on the β-face of the molecule as in compound **1-3**. The H_3-19 proton resonance showed a correlation with the H-10 (δ5.03, br s, $W_{1/2}$=19.9 Hz) proton resonance, indicating it was also in the β–orientation. The optical rotation for this compound was found to be -82.93. Compound **4** was identified as **(-)–**[1*R**,4*S**,10*R**]-4-hydroxycembra-2*E*,7*E*,11*Z*-trien-20,10-olide.

Compounds **1** and **3** were screened against the PEO1 and PEO1TaxR ovarian cancer cell lines and were found to have lower potency than paclitaxel (Table 2). However, sensitivity of taxane sensitive and taxane resistant cells was similar when treated with compounds **1** and **3**. This could point to a tubulin binding site that is different to that for paclitaxel. Moreover, the data imply that compounds 1 and 3 are not recognized by the multidrug resistance (MDR) transporter expressed in the PEO1TaxR cells, which is an important property that deserves further investigation. Experiments were repeated using stock solution which had been stored at 4^o for four days, and the decrease in activity demonstrated compound instability. Furanocembranoids from *Croton oblongifolius* have been shown to be active against BT474 (human breast ductol carcinoma), CHAGO (human undifferentiated lung carcinoma), Hep-G2 (human liver hepatoblastoma), KATO-3 (human gastric carcinoma), and SW-620 (human colon adrenocarcinoma) cell lines (Pudhom et al., 2007) and neocrotonal, isolated from the same source exhibited cytotoxicity against P-388 cells *in vitro* (Roengsumran *et al.*, 1999)

3. Experimental

3.1 *General experiment procedures*

1D and 2D NMR spectra were recorded in $CDCl_3$ on a 500 MHz Bruker AVANCE NMR (University of Surrey) instrument at room temperature. Chemical shifts (δ) are expressed in ppm and were referenced to the solvent resonances at 7.26 and 77.23 ppm for ^1H and ^{13}C NMR respectively. ESI mass spectra were recorded on a Bruker

MicroToF mass spectrometer using an Agilent 1100 HPLC to introduce samples (University of Oxford). Optical rotaions were determined in $CHCl_3$ on a JASCO P-1020 polarimeter (University of Surrey). FTIR spectra were recorded using a Perkin-Elmer (2000) spectrometer.

3.2 *Plant material*

Stem bark of *Croton gratissimus* Burch. var. *gratissimus* was collected from a mature tree cultivated on the campus of the University of KwaZulu-Natal, Durban, South Africa, identified by N.R. Crouch and a voucher retained for verification purposes (*Crouch 1051*, NH).

3.3 *Extraction and isolation of compounds*

The ground stem bark (1570 g) of *C. gratissimus* was extracted using a Soxhlet apparatus for 48 h successively using hexane, methylene chloride, ethyl acetate and methanol. After evaporation of the solvent *in vacuo* the following extracts were obtained: hexane (19.53 g), methylene chloride (18.29 g), ethyl acetate (20.26 g) and methanol (57.79 g). The hexane and methylene chloride extracts were examined in this study. Column chromatography over silica gel (Merck 9385) using a 5 cm diameter gravity column, collecting fractions (75 ml each) using a hexane/methylene chloride step gradient starting with 100% hexane and gradually increasing the methylene chloride concentration to 100%, followed by 5% methanol in methylene chloride, was used to separate the constituents. Final purification was undertaken using 1 cm diameter gravity column.

The hexane and methylene chloride extracts yielded compound **1** (Fraction 192, 6.1 mg, 50% hexane/50% CH_2Cl_2), **2,** (Fractions 18–25, 23.0 mg, 80% hexane/20% CH_2Cl_2) **3**, (Fraction 227, 12.0 mg, 100% CH_2Cl_2), **4**, (Fraction 145, 6.8 mg, 100% CH_2Cl_2), lupeol (Fraction 223, 20.3 mg, 100% CH_2Cl_2), 4(15)-eudesmene-1β,6α-diol (Fraction 227, 7.8 mg, 100 % CH_2Cl_2) and α-glutinol (Fraction 100, 15.0 mg, 100% CH_2Cl_2). The identity of 4(15)-eudesmene-1β,6α-diol and α-glutinol were confirmed by comparison of acquired NMR data against literature values (Sun *et al.,* 2004; Olea *et al*., 1993). NMR data for compounds **1**-**4** is given in Table 1.

3.3.1 *(+)–[1R*,2S*,7S*,8S*,12R*]–7,8-Epoxy-2,12-cyclocembra-3E,10Z-dien-20,10-olide* (**1**). White needle-like crystals; $[\alpha]_D^{23.6}$ +39.5 (*c* 0.0022, $CHCl_3$); IR ν_{max} (NaCl) cm^{-1} 3103, 2964, 2874 cm^{-1} (C – H stretches) and 1777 cm^{-1} (C = O stretch), ^1H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectroscopic data, see Table 1; HRESIMS *m/z* 339.1921 $[M + Na]^+$ (calc. for $C_{20}H_{28}O_3Na$ 339.193615).

3.3.2 *(+)–[1R*,10R*]-Cembra-2E,4E,7E,11Z-tetraen-20,10-olide* (**2**)
Colourless oil; $[\alpha]_D^{23.6}$ +23.77 (*c* 0.0042, $CHCl_3$); IR ν_{max} (NaCl) cm^{-1} 2952, 2918, 2845 (C – H stretch) and 1745 (C = O stretch), (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectroscopic data, see Table 1; HRESIMS *m/z* 323.1980 $[M + Na]^+$ (calc. for $C_{20}H_{28}O_2Na$ 323.19870).

3.3.3 *(+) –[1R*,4S*,10R*]-4-Hydroxycembra-2E,7E,11Z-trien-20,10-olide* (**3**)
Yellow oil; $[\alpha]_D^{23.6}$ +65.0 (*c* 0.0018, $CHCl_3$); IR ν_{max} (NaCl) cm^{-1} 3411 (O – H stretch) and 1738 (C = O stretch); ^1H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectroscopic data, see Table 1; HRESIMS *m/z* 341.2079 $[M + Na]^+$ (calc. for $C_{20}H_{30}O_3Na$ 341.209265).

3.3.4 *(-)–[1R*,4R*,10R*]-4-Hydroxycembra-2E,7E,11Z-trien-20,10-olide* (**4**)
Colourless oil; $[\alpha]_D^{23.6}$ -82.93 (*c* 0.0061, $CHCl_3$); IR ν_{max} (NaCl) cm^{-1} 3433 (O – H stretch) and 1736 (C = O stretch); ^1H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectroscopic data, see Table 1; HRESIMS *m/z* 341.2079 $[M + Na]^+$ (calc. for $C_{20}H_{30}O_3Na$ 341.209265).

3.4 *Single-crystal X-ray Analysis of* ***1***

Crystal data: $C_{20}H_{28}O_3$, space group $P2_12_12_1$, a= 6.198(2) Å, b= 11.676(5) Å, c=24.257(9) Å, V= 1755.5(11) $Å^3$, Z=4, D (calc) = 1.197 g/cm3, λ (Mo Kα)) 0. 71073 Å. Crystallographic data for compound **1** were collected at the University of Bristol X-ray Crystallography service a Bruker-Apex CCD diffractometer with Mo-K_a X-ray radiation (λ = 0.71073 Å) . Data were corrected for absorption using empirical methods (SADABS) (Bruker, 2004) based on symmetry-equivalent reflections combined with measurements at different azimuthal angles. The crystal structure was solved and refined by direct methods against all F^2 values using the

SHELXTL suite of programs and refined by least squares on weighted F^2 values for all reflections (see Supplementary Information 3) (Sheldrick, 2008). All non-hydrogen atoms were assigned anisotropic displacement parameters and refined without positional constraints. All the hydrogen atoms were located in the electron density difference map, and refined with appropriate distance restraints. The positions of the methyl hydrogen atoms were assigned by a rotating group refinement with fixed, idealised C-H distances. All other hydrogen atoms were constrained to ideal geometries. The hydrogen atoms were assigned isotropic displacement parameters equal to 1.5 times (methyl hydrogen atoms) or 1.2 times (all other hydrogen atoms) that of their parent atom. Refinement proceeded smoothly to give the residuals shown in Supplementary Information 3. A total of 1695 reflections were observed. In the absence of heavy atom in the structure, Friedel pairs were not collected, and the absolute configuration of the structure **1** was not determined.

3.5 *Screening*

All tissue culture reagents were obtained from Sigma Aldrich (Poole, UK), unless stated otherwise. The PEO1 ovarian cancer cell line was originally developed by Langdon et al. and obtained from Prof F. Balkwill (formerly of ICRF laboratories, Lincoln Inn Fields, London, UK). The PEO1TaxR (paclitaxel resistant) cell line was derived in-house and possesses approximately 12- fold resistance to its inducing agent (referred to in Coley *et al.,* 2006). PEO1 cell lines were cultured as monolayers in RPMI-1640 medium supplemented with 10% foetal calf serum (heat inactivated, obtained from Invitrogen, Paisley, UK) and 2 mM Glutamax (Invitrogen). Cells in monolayer culture were subjected to trypsinisation (trypsin-EDTA solution; Sigma Aldrich) prior to use in cytotoxicity testing (see below).

Working stock solutions of compounds **1** and **3** were prepared by dissolving compounds in chloroform to give a concentrated solution of 50 mM. Cytotoxicity was determined by means of the colorimetric assay MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Cultured cell monolayers were reduced to a single cell suspension (as described above) and then seeded into 96-well tissue culture plates at a density of 6 x 10^3 cells per well for all cell lines. This was calculated to allow for exponential growth of the cultures throughout the incubation period. Cells were allowed to settle for 24 h, under standard culture incubation conditions and then drug

treated in quadruplicate with a dose range spanning 2-logs of drug concentration, each drug aliquot being administered in a 50 μl volume. All drug dilutions were carried out in complete culture medium. After 72 h incubation under standard culture conditions MTT solution (5 mg/ml in PBS) was added in a 20 μl volume and incubated for a further 4 h. The MTT/medium mixture was then removed and the resulting formazan crystals dissolved in 200 μl of DMSO. The optical density of the purple color product was measured at 550 nm in a plate reading spectrophotometer. The quantity of live cells was expressed as T/C values by comparison with untreated control microcultures. The concentration of complexes that decreased absorption by 50% were calculated by interpolation and expressed as IC_{50} values.

4. Supplementary information

Supplementary data associated with this article can be found, in the online version, XXXXXXXXXXX. Crystallographic data for structure **1** is deposited at the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 764183. Copies of the data can be obtained free of charge on application to CCDC, 12 UnionRoad, Cambridge CB2 1EZ, UK (Fax: + 44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk).

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5. References

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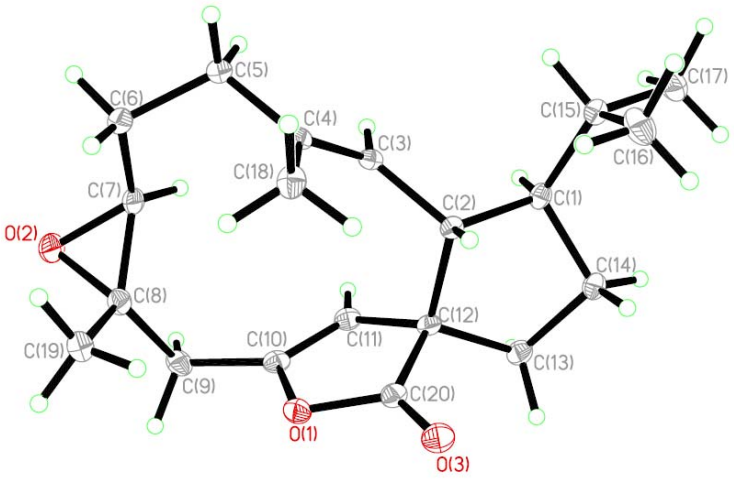

Figure 1: ORTEP illustration for compound 1: (+)–[1*R**,2*S**,7*S**,8*S**,12*R**]–7, 8-Epoxy-2,12-cyclocembra-3*E*,10*Z*-dien-20,10-olide

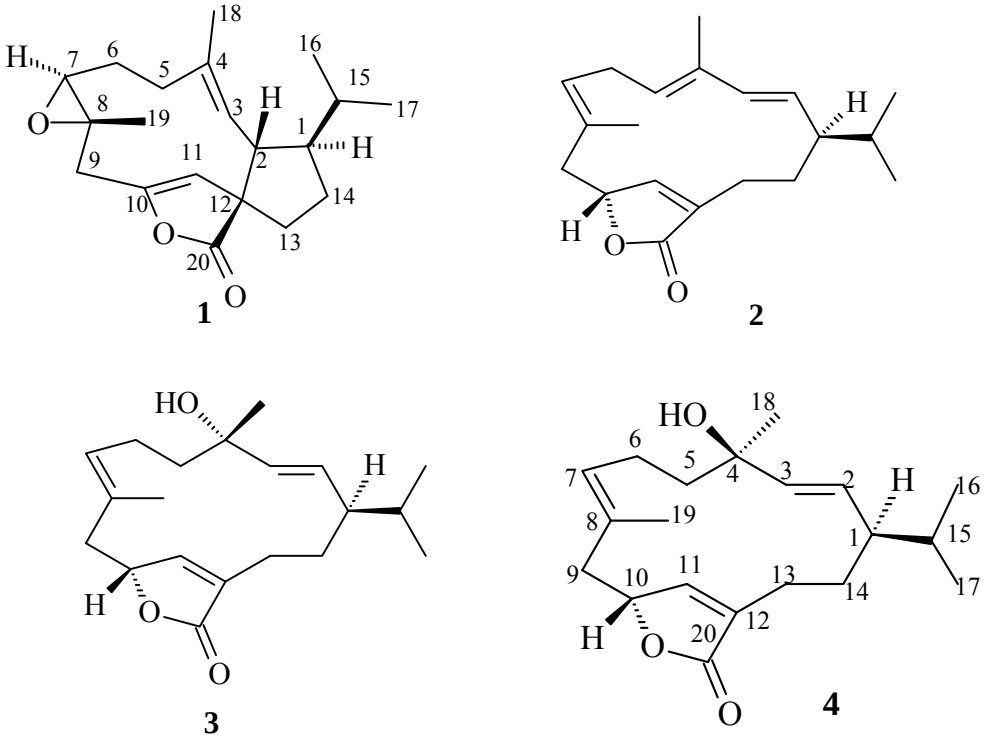

Figure 2: Structures of compounds **1-4.**

Table 1: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) chemical shifts in $CDCl_3$ for compounds 1 - 4

	1		2		3		4	
No	**^{13}C NMR**	**^{1}H NMR**	**^{13}C NMR**	**^{1}H NMR**	**^{13}C NMR**	**^{1}H NMR**	**^{13}C NMR**	**^{1}H NMR**
1	54.0 CH	1.70 m	50.1 CH	1.72 m	48.4 CH	1.58 m	48.7 CH	1.58 m
2	54.8 CH	2.91 t J = 9.6 Hz	130.2 CH	5.26 dd J = 15.4, 10.0 Hz	131.4 CH	5.41 m	130.5 CH	5.35 t J = 7.3 Hz
3	128.5 CH	4.85 d J = 9.6 Hz	130.8 CH	6.00 d J = 15.4 Hz	138.0 CH	5.60 d J = 15.7 Hz	138.6 CH	5.50 d J = 16.9 Hz
4	136.0 C	-	135.3 C	-	72.5 C	-	73.6 C	-
5α	38.5 CH_2	2.23 dd J = 3.9, 8.8 Hz	125.1 CH	5.49 t J = 7.5 Hz	43.7 CH_2	1.92 m	43.8 CH_3	1.85 m
5β						1.51 m		1.58 m
6α	23.2 CH_2	1.87 m	26.4 CH_2	2.41 m	25.6 CH_2	2.34 m	25.7 CH_2	2.40 m
6β		1.57 m		3.05 m		1.59 m		2.30 m
7α	63.0 CH	2.64 d J = 9.9 Hz	129.1 CH	5.10 d J = 9.6 Hz	131.1 CH	5.37 t J = 7.5 Hz	129.5 CH	5.51 m
8	61.9 C	-	128.1 C	-	129.4 C	-	129.9 C	-
9α	39.0 CH_2	1.98 d J = 13.5 Hz	41.9 CH_2	2.46 m	44.7 CH_2	2.07 dd J = 2.6,12.7 Hz	44.6 CH_2	2.10 dd J = 10.2, 12.6 Hz
9β		2.86 q J = 4.3 Hz		2.78 d J = 14.6 Hz		2.80 dd J = 7.3,12.7 Hz		2.81 dd J = 5.4, 12.6 Hz
10	151.2 C	-	81.8 CH	5.04 br s $W_{1/2}$ = 8.1 Hz	80.3 CH	5.03 br s $W_{1/2}$ = 19.6 Hz	80.3 CH	5.03 br s $W_{1/2}$ = 19.9 Hz
11	110.6 CH	5.05 br s $W_{1/2}$ = 2.8 Hz	151.3 CH	6.80 s	149.6 CH	6.98 d J = 1.6 Hz	149.5 CH	7.02 d J = 1.7 Hz
12	58.3 C	-	132.5 C	-	133.6 C	-	133.9 C	-
13α	33.3 CH_2	1.78 m	25.1 CH_2	2.33 m	22.7 CH_2	2.20 m	23.4 CH_2	2.00 m
13β		2.14 m						2.35 m
14α	28.2 CH_2	1.84 m	26.5 CH_2	2.41 m	28.1 CH_2	1.94 m	28.7 CH_2	1.85 m
14β		1.67 m		1.38 m		1.49 m		1.58 m
15	29.9 CH	1.68 m	33.0 CH	1.55 m	33.9 CH	1.60 m	34.0 CH	1.58 m
16	18.8 CH_3	0.85 d J = 6.6 Hz	20.0 CH_3	0.82 d J = 6.8 Hz	19.6 CH_3	0.84 d J = 6.5 Hz	19.5 CH_3	0.82 d J = 6.4 Hz
17	22.0 CH_3	0.92 d J = 6.6 Hz	20.9 CH_3	0.89 d J = 6.8 Hz	20.4 CH_3	0.89 d J = 6.5 Hz	20.6 CH_3	0.88 d J = 6.4 Hz
18	15.1 CH_3	1.52 d J = 1.2 Hz	19.6 CH_3	1.78 s	28.2 CH_3	1.37 s	30.3 CH_3	1.30 s
19	19.2 CH_3	1.42 s	18.0 CH_3	1.70 s	16.5 CH_3	1.67 s	16.5 CH_3	1.67 s
20	183.6 C	-	174.0 C	-	174.2 C	-	174.2 C	-

Table 2: Chemosensitivity testing in taxane sensitive (PEO1) and resistant PEO1TaxR human ovarian cancer cells (data shown are IC_{50} drug concentrations in nM amounts) for compounds 1 and 3.

Compound	PEO1	PEO1TaxR	Resistance Factor
1	132	200	1.5
3	125	135	< 1.0
Paclitaxel	2.3	30.5	13.3

SUPPLEMENTARY INFORMATION 1

Table 1. Atom and NMR correlation description of compound **1**, used as input to the LSD structure generator.

LSD Atom #	Status[a]			HMBC correlation[b]	Atom # in **1**
1	C	sp^2	0	5, 9, 13	20
2	C	sp^2	0	5, 11	10
3	C	sp^2	0	9, 12, 20	4
4	C	sp^2	1	9, 12, 20	3
5	C	sp^2	1	9, 11, 13	11
6	C	sp^3	1	11, 12, 18	7
7	C	sp^3	0	6, 11, 16, 18	8
8	C	sp^3	0	5, 9, 13	12
9	C	sp^3	1	–	2
10	C	sp^3	1	4, 9, 17, 19	1
11	C	sp^3	2	18	9
12	C	sp^3	2	4, 6, 16	5
13	C	sp^3	2	–	13
14	C	sp^3	1	9, 19	15
15	C	sp^3	2	13	14
16	C	sp^3	2	6, 12	6
17	C	sp^3	3	19	16
18	C	sp^3	3	11	19
19	C	sp^3	3	17	17
20	C	sp^3	3	4, 12	18
21	O	sp^2	0	–	–
22	O	sp^3	0	–	–
23	O	sp^3	0	–	–

[a]The status is defined by the element, hybridization state, multiplicity triplet. [b]Hydrogen atom numbers.

SUPPLEMENTARY INFORMATION 2

Computer file for the LSD-assisted structure elucidation of compound **1**.

```
; Compound 1
; Cembranoid from
; Croton gratissimus

; CDCl3 77.233

; Atom 1 is a C, sp2,
; 0 H attached.
MULT 1 C 2 0 ; 183.62
MULT 2 C 2 0 ; 151.16
MULT 3 C 2 0 ; 135.96
MULT 4 C 2 1 ; 128.50
MULT 5 C 2 1 ; 110.65
MULT 6 C 3 1 ; 63.01
MULT 7 C 3 0 ; 61.92
MULT 8 C 3 0 ; 58.32
MULT 9 C 3 1 ; 54.82
MULT 10 C 3 1 ; 53.97
MULT 11 C 3 2 ; 39.03
MULT 12 C 3 2 ; 38.48
MULT 13 C 3 2 ; 33.33
MULT 14 C 3 1 ; 29.92
MULT 15 C 3 2 ; 28.17
MULT 16 C 3 2 ; 23.19
MULT 17 C 3 3 ; 22.02
MULT 18 C 3 3 ; 19.22
MULT 19 C 3 3 ; 18.75
MULT 20 C 3 3 ; 15.15
MULT 21 O 2 0
MULT 22 O 3 0
MULT 23 O 3 0

; C4 is bonded to H4
HMQC 4 4
HMQC 5 5
HMQC 6 6
HMQC 9 9
HMQC 10 10
HMQC 11 11
HMQC 12 12
HMQC 13 13
HMQC 14 14
HMQC 15 15
HMQC 16 16
HMQC 17 17
HMQC 18 18
HMQC 19 19
HMQC 20 20



; C1 correlates
; with H5 in HMBC
HMBC 1 5
HMBC 1 9
HMBC 1 13
HMBC 2 5
HMBC 2 11
HMBC 3 9
HMBC 3 12
HMBC 3 20
HMBC 4 9
HMBC 4 12
HMBC 4 20
HMBC 5 9
HMBC 5 11
HMBC 5 13
HMBC 6 11
HMBC 6 12
HMBC 6 18
HMBC 7 11
HMBC 7 6
HMBC 7 16
HMBC 7 18
HMBC 8 5
HMBC 8 9
HMBC 8 13
HMBC 10 4
HMBC 10 9
HMBC 10 17
HMBC 10 19
HMBC 11 18
HMBC 12 4
HMBC 12 6
HMBC 12 16
HMBC 14 9
HMBC 14 19
HMBC 15 13
HMBC 16 6
HMBC 16 12
HMBC 17 19
HMBC 18 11
HMBC 19 17
HMBC 20 4
HMBC 20 12

; L1: quaternary C
QUAT L1

; L2: methine C
CH   L2
; Atoms 17 and 19 are
; bonded to a methine C
PROP 17 1 L2
PROP 19 1 L2

; Atoms 18 and 20 are
; bonded to a quat. C
PROP 18 1 L1
PROP 20 1 L1

; the carbonyl group
BOND 1 21

; L7: atoms with
; all carbon neighbours
LIST L7 13 14 15 16 22 23

; L8: all carbon atoms
CARB L8
; Each atom in L7
; has all (0) its
; neighbours in L8.
; Next line may be
; commented (;)
PROP L7 0 L8

; remove substructure
; constraint.
; Next line may be
; commented (;)
SUBS 0

; Cyclopropane description
; Sub-atom S1 is a C, sp3,
; with 0 or 1 or 2 H
; atom attached
SSTR S1 C 3 (0 1 2)
SSTR S2 C 3 (0 1 2)
SSTR S3 O 3 0

; Sub-atom bonds
LINK S1 S2
LINK S1 S3
LINK S2 S3
```

SUPPLEMENTARY INFORMATION 3

Crystal data and intensity collection for compound 1

Empirical formula	$C_{20}\,H_{28}\,O_3$
Formula weight	316.42
Temperature	100(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	P 21 21 21
Unit cell dimensions	a = 6.198(2) Å α = 90°
	b = 11.676(5) Å β = 90°
	c = 24.257(9) Å δ = 90°
Volume	1755.5(11) $Å^3$
Z	4
Density (calculated)	1.197 Mg/m^3
Absorption coefficient	0.079 mm^{-1}
$F(000)$	688
Crystal size (mm)	0.247 x 0.189 x 0.06
θ range for data collection	1.68 to 26.56°
Index ranges	$-7 \le h \le 7$, $-14 \le k \le 14$, $-30 \le l \le 30$
Reflections collected	11828
No. of Friedel pairs	1695
Independent reflections	2113 [R_{int} = 0.0704]
Completeness to θ =28.56°,	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.993 and 0.982
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2136 / 0 / 212
Goodness-of-fit on F^2 S	1.042
R indices [for 1665reflections, $I>2\delta(I)$]	R_1 = 0.0386, wR_2 = 0.0801
R indices (for all 2136 data)	R_1 = 0.0587, wR_2 = 0.0878
Absolute structure (Flack) parameter	0.03(2)
Largest diff. peak and hole	0.183 and -0.181 $eÅ^{-3}$