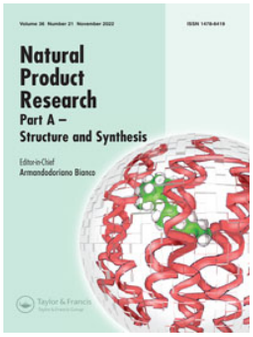

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Anti-HIV crotocascarin ω from Kenyan *Croton dichogamus*

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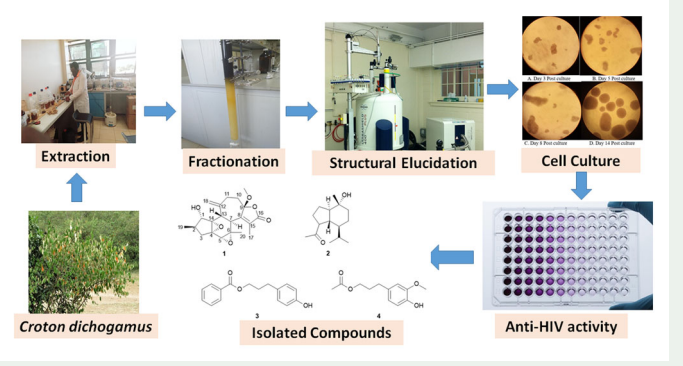
ABSTRACT

An anti-HIV methanol-soluble fraction of a 1:1 CH_2Cl_2:CH_3OH extract of twigs of a Kenyan *Croton dichogamus* yielded seven compounds, the new crotocascarin ω (**1**), the known β-oplopanone (**2**), dihydroconiferyl acetate (**3**), 3′(4″-hydroxyphenyl)-propyl benzoate (**4**), lupeol, sitosterol and stigmasterol. Crotocascarin ω (90%) inhibited HIV-1 replication with an IC_{50} value of 5.3 nM, and the compound was cytotoxic towards MT-4 cells presenting an IC_{50} value of 84 μM. *In silico* modelling showed that the anti-HIV activity for compound **1** could be through the HIV-1 protease inhibition.



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Anti-HIV activity; *Croton dichogamus*; crotocascarin ω; β-oplopanone; dihydroconiferyl acetate; 3′(4″-hydroxyphenyl)-propyl benzoate

1. Introduction

The anti-HIV potential of several members of the *Croton* genus has been documented, and compounds with anti-HIV activity have been previously reported from *Croton echinocarpus* (Ravanelli et al. 2016), *Croton megalobotrys* (Tietjen et al. 2016, 2018) and *Croton tiglium* (El-Mekkawy et al. 2000). In a recent study, we also demonstrated that crude extracts from three Kenyan *Croton* plants, *Croton dichogamus, Croton*

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macrostachys and *Croton megalocarpus* (Terefe et al. 2021) have anti-HIV potential. In addition, we recently reported anti-HIV potential of crotofolanes from *C. megalocarpus* (Terefe et al., 2022a). In this study, we report anti-HIV activity of a crotofolane diterpenoid, crotocascarin ω, from *C. dichogamus*.

Croton dichogamus Pax (Euphorbiaceae) is a shrub or small tree of the genus *Croton* (Euphorbiaceae), with more than 1300 species that occur in the tropical and sub-tropical regions of the world. *C. dichogamus* grows in eastern Africa, in Somali, Ethiopia, Kenya, Tanzania and Mozambique, and in Madagascar. In the East African countries, decoctions of leaves, roots, and whole plants are used to treat fever, chest ailments, stomach diseases, tuberculosis, impotence, and malaria (Mohagheghzadeh et al., 2006; Jeruto et al., 2008; Owuor et al., 2012; Terefe et al., 2022b) . In Tanzania, the roots of *C. dichogamus* are milled and then mixed with porridge for the treatment of tuberculosis because the shrub is believed to be efficient in the management of respiratory ailments. The leaves are also used as a tonic, antimalarial and nutritional supplement. Patients inhale the smoke of burnt leaves to provide relief from fever (Terefe et al., 2022c). The ethanol extract of roots of *C. dichogamus* has demonstrated antimycobacterial activity against *M. indicus pranii* giving a minimum inhibitory concentration (MIC) of 1.25 mg/mL and *M. madagascariense*, also giving a minimum inhibitory concentration (MIC) of 1.25 mg/mL (Magadula, 2012). *C. dichogamus* has also been showed to have insecticidal activity against *Anopheles gambie* and is extensively used to treat malaria in lake basins of East Africa (Omara 2020). Previously the chemistry of *C. dichogamus*, collected in Kenya, led to the identification of crotofolane diterpenoids, crotoxide A and B (Jogia et al. 1989), crotodichogamoin A and B (Aldhaher et al. 2017), and 15,16-epoxy-3-hydroxy-5(10), 13(16), 14-*ent*-halimatriene-17, 12(*S*)-olide, 15,16-epoxy-5,13(16),14-*ent*-halimatriene-3-ol, and 1,3,5-cadinatriene-(7*R*),(10*S*)-diol, 15,16-epoxy-4(18),13(16),14-*ent*-clerodatrien-3α-ol (gbaninol), crotohaumanoxide, furocrotinsulolide A, *trans*-cascarillone, 3β,4β,15,16-diepoxy-13(16),14-clerodadiene, cyperene, cadalene, 4α,5β-corocalanediol, 10-*epi*-maninsigin D and aleuritolic acid. These compounds were evaluated against caco-2 cell lines and the NCI69 cancer cell line screen at a concentration of 1×10^{-5} M, but they did not show significant cytotoxicity (Aldhaher et al. 2017). The current study examined the anti-HIV potential of *C. dichogamus*. As part of our continued research on useful compounds from African *Croton* species (Langat et al. 2011; Ndunda et al. 2013, 2016; Isyaka et al. 2020; Langat et al. 2018, 2020), we conducted an extensive chemical analysis of *C. dichogamus*, the anti-HIV and cytotoxic effects. We demonstrated that crotocascarin ω isolated from methanol-soluble fraction of a 1:1 CH_2Cl_2:CH_3OH extract of twigs of a Kenyan *C. dichogamus* exhibited anti-HIV and cytotoxic effect.

2. Result and discussion

2.1. Structural elucidation of crotocascarin ω (1)

Compound **1** was isolated from the methanol-soluble fraction of a 1:1 CH_2Cl_2:CH_3OH extract of twigs of *C. dichogamus* and identified as an undescribed epimer of the known crotocascarin M (**1a**) previously isolated from *Croton cascarilloides* (Figure 1) (Kawakami et al. 2015, 2016). Therefore, the compound was assigned as crotocascarin ω. Compound (**1**) has a molecular formula of $C_{21}H_{26}O_6$, with nine degrees of unsaturation, as

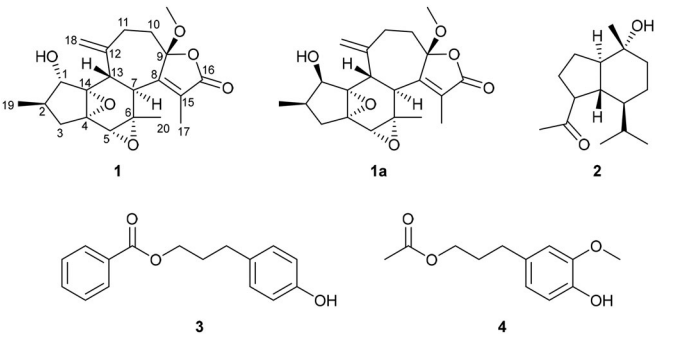

Figure 1. Crotocascarin ω (**1**), β-oplopanone (**2**), dihydroconiferyl acetate (**3**), and 3′(4′′-hydroxyphenyl)-propyl benzoate (**4**) isolated from the methanol soluble fraction of a 1:1 CH_2Cl_2:CH_3OH extract of twigs of *C. dichogamus* and the known crotocascarin M (**1a**).

determined from (+)-HRESIMS analysis, which displayed an ion peak at *m/z* 375.1799 $[M+H]^+$ (calcd for $[C_{21}H_{27}O_6]^+$, *m/z* 375.1802). The IR spectrum exhibited the presence of an α,β-unsaturated lactone (1733 cm^{-1}) and hydroxy groups (3429 cm^1). The ^{1}H NMR spectrum showed four methyl resonances, including a doublet resonance, downfield resonances for the methyls of a methoxy, and an acetoxy and an allylic methyl resonance, a hemiketal resonance, five oxygenated methine resonances, and exocyclic methylene proton resonances. The ^{13}C NMR spectrum showed 21 carbon resonances, including a hemiketal carbon resonance at 109.6 (Figures S1–S9). The use of HMBC and COSY showed that compound **1** was an isomer of the known crotocascarin M (Kawakami et al. 2016), with differences in the ^{13}C NMR chemical shifts for C-1 (75.7 for compound **1** and 73.7 for crotocascarin M), C-3 (33.3 for compound **1** and 36.3 for crotocascarin M (**1a**)), C-4 (62.9 for compound **1** and 60.3 for crotocascarin M), C-5 (56.2.7 for compound **1** and 58.2 for crotocascarin M), C-6 (63.3 for compound **1** and 56.6 for crotocascarin M), C-11 (33.1 for compound **1** and 34.9 for crotocascarin M), C-12 (145.8 for compound **1** and 147.7 for crotocascarin M), (45.8 for compound **1** and 39.5 for crotocascarin M) and C-14 (73.3 for compound **1** and 70.7 for crotocascarin M) (Table S1). In addition, ^{1}H NMR showed differences for H-5, H-7, 2H-10, 2H-11, H-3, 3H-17, 2H-18, 3H-19, and 1-OH between compound **1** and crotocascarin M. The NOESY spectrum showed that H-1, H-5, H-13, 9-OCH_3, 3H-19, and 3H-20 were on one face, whereas 1-OH and H-7 were on the other face (Figure S7). The data described above supported the assignment of **1** as a 1α-hydroxy derivative of the known crotocascarin M (**1a**) named crotocascarin ω (Kawakami et al. 2015, 2016).

The known β-oplopanone (**2**) (Jung et al. 1997), dihydroconiferyl acetate (**3**) (Kondo et al. 2007), 3′(4′′-hydroxyphenyl)-propyl benzoate (**4**) (Athikomkulchai et al. 2006), lupeol, sitosterol and stigmasterol were also identified from the twigs of *C. dichogamus* (Figure 1).

2.2. Cytotoxicity and anti-HIV activity of crotocascarin ω (1)

Compound **1** (>90%) showed significantly ($p < 0.01$) higher maximum cytotoxic effect at 84 μM and with a high $Emax_c$ when compared with the control drugs TDF and ABC (Table 1). Compound **1** displayed the highest anti-HIV activity by inhibiting viral

Table 1. Cytotoxicity and antiretroviral activity of crotocascarin ω (**1**) and methanol-soluble fraction of *C. dichogamus*.

	Cytotoxicity			Antiviral activity		
Materials	MNTC (μg/mL)	CC_{50} (μg/mL)	$Emax_C$ (%)	IC_{50} (μg/mL)	$Emax_{AV}$ (%)	SI
AZT	0.38 ± 0.19	0.53 ± 0.29	36.28 ± 0.83	0.002 ± 0.00	83.5 ± 0.57	279.4
TDF	4.92 ± 0.71	6.73 ± 0.24	13.17 ± 0.43	0.04 ± 0.01	80.55 ± 0.46	176.5
ABC	0.18 ± 0.03	0.26 ± 0.00	17.83 ± 0.57	0.05 ± 0.031	58.67 ± 0.43	5.0
NVP	0.57 ± 0.0	0.82 ± 0.0	39.13 ± 0.65	0.24 ± 0.09	72.53 ± 0.47	3.5
CDM	15.4 ± 0.45	19.58 ± 0.79	42.2 ± 0.62	0.06 ± 0.01	90.83 ± 0.18	318.5
1	16.36 ± 0.29	31.46 ± 0.51	55.03 ± 5.11	0.002 ± 0.01	76.19 ± 0.01	15429.1

Results are shown as means ± S.E. M (n = 3) AZT, Zidovudine; TDF, Tenofovir; ABC, Abacavir; NVP, Nevirapine; CDM, *C. dichogamus* twigs methanol-soluble extract; MNTC, Maximum non-toxic concentration; CC_{50}, 50% cytotoxic concentration; $Emax_C$, Maximum cytotoxic effect %; IC_{50}, 50% antiviral effect concentrations; $Emax_{AV}$, maximum antiviral effect %; SI, selective index.

replication at the lowest IC_{50} value of 0.002 ± 0.01 μg/mL (5.3 nM) (Figures S10 and S11). A synergistic effect of the two compounds cannot be discounted.

2.3. Molecular docking results for crotocascarin ω (1)

To propose a mode-of-action of compound **1**, in silico inhibition modelling assays were carried out using HIV reverse transcriptase and HIV protease. Docking studies were performed on HIV-1 RT in complex with known inhibitor nevirapine (PDB ID 1JLB) as well as HIV-1 PR in complex with known antiviral atazanavir (PDB ID: 3EL9) using MOE2015 software (Figure S12). The predicted free energy of binding obtained for compound 1 against HIV-1 RT was higher (ΔG −1.38 kcal/mol) compared to the known inhibitor nevirapine (ΔG −7.60 kcal/mol), due to the lack of the crucial π-H interaction shown by Nevirapine. The main interaction contributing to the binding was the hydrogen bond between hydroxy group at C-1 and Cys181 (Figure S13). For HIV-PR, the predicted free energy of binding exhibited by compound 1 was ΔG −6.25 kcal/mol, which was also higher compared to the positive control atazanavir (ΔG −11.49 kcal/mol). The main interaction contributing to the binding was the hydrogen bonding between one of the epoxide oxygens and the backbone N-H of Asp29(B). Asp29(B) also shows a key hydrogen bonding interaction with one of the amide carbonyls of Atazanavir (Figure S14). Protease inhibitors usually contain a hydroxyethylene core which mimics the transition state of the hydrolysis step by binding with the catalytic aspartic residues Asp29(B) (Ghosh et al. 2016; Figure S14). Therefore, the residues of HIV protease that interact with the inhibitors, such as Gly-27, Asp-29, Asp-30, and Gly-48, are highly conserved (Lv et al., 2015).The formation of hydrogen bonds with these residues at the catalytic region will impair the enzyme's activity. In biological complexes, hydrogen bonds are the most common directed intermolecular interactions, and they contribute significantly to the specificity of molecular recognition. With these results it can be hypothesised that the potential anti-HIV activity of crotocascarin ω could be caused by HIV-1 PR inhibition.

As a consequence, the motifs present in crotocascarin ω could be used as a starting point to maximise binding interactions with the S2 subsite containing Asp25, but the structure should be modified to interact with the rest of the active site in order to be considered as a potential HIV-PR inhibitor.

3. Experimental

The twigs of *C. dichogamus* were collected and extracted as described in our earlier work (Terefe et al. 2022). The methanol soluble fraction of a 1:1 CH_2Cl_2:CH_3OH extract of twigs of *C. dichogamus* was subjected to column chromatography at the Department of Pharmacology and Pharmacognosy, United States International University, Kenya. Commercial silica gel (100 − 200, 200 − 300, and 300 − 400 mesh; Qingdao, China) was used for column chromatography (CC). Sephadex LH-20 (Amersham Biosciences) was also used for CC. All solvents used for column chromatography were of analytical grade (Shanghai Chemical Reagents Co., Ltd.). Analytical TLC using precoated aluminum-backed plates (silica gel 60 F_{254}, Merck) was used. Spots were detected on TLC under UV light at 254 or 365 nm, followed by spraying with 1% vanillin-sulfuric acid spray reagent and warming. 1 D and 2 D NMR spectra were recorded in $CDCl_3$ on a 400 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts (δ) are expressed in ppm and were referenced against the solvent resonances at δ_H 7.26 and δ_C 77.23 ppm for ^1H and ^{13}C NMR for $CDCl_3$. Structural assignments of the new compounds were made with additional information from ^1H-^1H COSY, HSQC, NOESY, and HMBC experiments. Mass spectra were recorded on a GC-MS Bruker MicroToF Mass Spectrometer by direct injection using a Bruker Bioapex-FTMS with electrospray ionisation. The above analysis was performed at the Jodrell Laboratory, Royal Botanic Gardens Kew (UK).

Crotocascarin ω (**1**). White oil; [α] +28.7 (*c* 0.03, $CHCl_3$); IR (neat) ν_{max} 3429, 1733 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): 5.28 (br, s, H-18A), 5.16 (br, s, H-18B), 4.16 (dd, 4.6, 4.6, H-1), 3.40 (s, 9-OCH_3), 3.15 (d, 0.6, H-5), 2.96 (dd, 1.4, 12.6, H-7), 2.91 (d, 12.6, H-13), 2.65 (m, H-10α), 2.33 (m, H-11α), 2.27 (m, H-11β), 2.18 (dd, 11.5, 6.1, H-3α), 1.95 (m, H-2), 1.94 (d, 1.3, H_3-17), 1.68 (dd, 11.5, 13.7, H-3β), 1.61 (m, H-10β), 1.29 (d, 4.9, 1-OH), 1.16 (s, H_3-20) and 0.98 (d, 7.1, H_3-19 and ^{13}C NMR ($CDCl_3$, 100 MHz): 171.1 (C-16), 159.4 (C-8), 145.8 (C-12), 129.0 (C-15), 114.6 (C-18), 109.6 (C-9), 75.5 (C-1), 73.3 (C-14), 63.3 (C-4), 62.9 (C-6), 56.2 (C-5), 51.9 (9-OCH_3), 45.8 (C-7), 44.5 (C-13), 35.8 (C-10), 33.5 (C-2), 33.3 (C-3), 33.1 (C-11), 20.1 (C-20), 12.2 (C-19) and 10.1 (C-17; HRESIMS *m/z* 375.1799 $[M + H]^+$ (calcd. for $C_{21}H_{27}O_6^+$, *m/z* 375.1808).

The human T-lymphocytic MT-4 cells (ARP-120) were obtained through the National Institute of Health (NIH) HIV Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH: MT-4 Cells, ARP-120, contributed by Dr. Douglas Richman. Human immunodeficiency virus type 1 (HIV-1) IIIB (also referred to as HTLV-IIIB) was obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH: Human Immunodeficiency Virus-1 IIIB, ARP-398, contributed by Dr. Robert Gallo. The cytotoxicity test was conducted by measuring cell death caused by the test compounds. The assay was conducted using MTT colorimetric assay as described previously by Mosmann and Pauwels (Mosmann 1983; Pauwels et al. 1988). The MTT assay was based on the reduction of the yellow-colored tetrazolium salt MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltet diphenyltetrazolium bromide) by NAD(P)H-dependent cellular oxidoreductase enzymes to an insoluble dark-blue colored formazan that can be measured spectrophotometrically (Berridge et al. 2005). More details on the protocol followed for this assay is provided as supplementary material.

The docking studies were performed on MOE2015 software package using HIV-1 reverse transcriptase (HIV-1 RT) in complex with known inhibitor nevirapine (PDB ID: 1JLB) and wild-type HIV-1 protease complexed with known antiviral atazanavir (PDB ID: 3EL9). The proteins were prepared by first removing all water molecules, and in the case of HIV-1 PR also the sulfate and formate ions present in the PDB file. Then, hydrogens were added, and the structures were protonated. For the ligand, energy minimisation was performed using molecular mechanics forcefield MMFF94x. To validate the docking protocol used, known inhibitors nevirapine and atazanavir were removed from their corresponding binding pockets and redocked. The Root Mean Square Deviation (RMSD) value from the known co-crystallised conformation was 0.0551 Å and 1.6038 Å respectively. Compound **1** was docked using MOE2015 with triangle matcher, scoring by London dG, 100 poses as placement method and rigid receptor, GBVI/WSA dG 5 poses as refinement method in both targets. The docking procedure was repeated in three independent runs. The lowest scoring affinity pose in each ligand was used to study the ligand interactions (Rotich et al. 2021).

4. Conclusions

We conclude that crotocascarin ω (**1**) is a potent anti-HIV compound. This compound is from the rare crotofolane diterpenoid class, which possess a fused 5-, 6- and 7-membered rings, biosynthesised from cembranes via casbane, and lathyrane through cross annular cyclisation (Kawakami et al. 2015). Therefore, there is need to subject the over 38 crotofolanes from the *Croton* genus to anti-viral assays.

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Institutional review board statement

The collection of the plant was performed after obtaining the required ethical approval from the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (KNH-UON ERC), approval number P992/12/2019.

Disclosure statement

No potential conflict of interest was reported by the authors.

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