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Diterpenes Isolated from *Croton zambesicus* Inhibit KCl-Induced Contraction

Abstract

A mixture of two new diterpenes was isolated from a dichloromethane extract of *Croton zambesicus*: *ent*-18-hydroxytrachyloban-3β-ol (**1**) and *ent*-18-hydroxyisopimara-7,15-diene-3β-ol (**2**). The two compounds crystallised together and were separated after derivatisation of the pimarane derivative with osmium tetroxide. The structure of **1** was elucidated by 1D- and 2D-NMR analysis and by X-ray diffraction of a crystal containing both compounds while **2** was only identified by crystallographic data. As this plant is widely used in African folk medicine against hypertension, we have analysed the vasorelaxant activity of the isolated molecules. The mixture of the two compounds inhibited the KCl-induced contraction of male Wistar rat aorta (IC_{50} = 1 μg/mL), while the purified trachylobane (compound **1**) and the hydroxylated pimarane showed a lower activity than the mixture.

Key words

Croton zambesicus · *ent*-18-hydroxytrachyloban-3β-ol · *ent*-18-hydroxyisopimara-7,15-diene-3β-ol · vasorelaxant activity

Introduction

Croton zambesicus Muell. Arg. is a plant widely used in African traditional medicine. Phytochemical studies have shown the presence of clerodanes, labdanes and trachylobanes in the stem bark [1], [2]. More recently, trachylobanes (*ent*-trachyloban-3β-ol, *ent*-trachyloban-3-one, *ent*-18-hydroxytrachyloban-3-one) possessing moderate cytotoxic activity on HeLa cells (IC_{50} = 7.3, 12.2, 9.6 μg/mL, respectively) were isolated from the leaves of *C. zambesicus* [3], [4].

In the present work, we report the characterisation of two new diterpenes: *ent*-18-hydroxytrachyloban-3β-ol (**1**) and *ent*-18-hydroxyisopimara-7,15-diene-3β-ol (**2**) isolated from the dichloromethane extract of the *C. zambesicus* leaves (Fig. **1**).

As the leaves of *Croton zambesicus* are used in African folk medicine to treat hypertension [5], we tested whether these compounds could behave as Ca^{++} channel blockers and inhibit KCl-evoked contractions, and evaluated their cytotoxicity.

Materials and Methods

General experimental procedure

High speed counter-current chromatography was performed on an HSCCC Kromaton III, SEAB. An Omnifit glass column (OM 6427 15×750 mm) packed with Lichroprep Si 60 (15–25 μM, Merck) was used for MPLC. Analytical TLC was performed on precoated silica gel 60 F_{254} plates (Merck) and detection was achieved by spraying with sulphuric acid-anisaldehyde, followed

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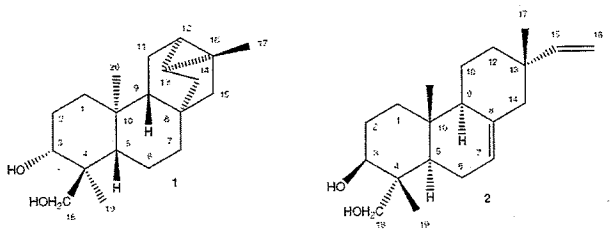

Fig. 1 Structures of *ent*-18-hydroxytrachyloban-3β-ol (**1**) and 18-hydroxyisopimara-7,15-dien-3β-ol (**2**).

by heating 5 min at 105 °C. NMR spectra were recorded on a Bruker Avance 500 spectrometer in $CDCl_3$ with TMS as internal reference. MS analyses were achieved using electronic impact ionisation (EI-MS, 70eV) on a Finnigan TSQ700 triple quadrupole instrument.

Plant material

The aerial parts of *Croton zambesicus* were collected in the Cotonou area (Benin) and identified by Prof. V. Adjakidje (Université d'Abomey-Calawi, Bénin). A voucher has been deposited at the herbarium of Belgian National Botanical Garden at Meise (BR S.P. 848.108).

Extraction and isolation

580 g of air-dried powdered leaves were macerated (1800 mL of CH_2Cl_2 during 48 h) and then extracted by percolation (3200 mL of CH_2Cl_2 at room temperature) to give 34 g of final extract. 5 g of this extract were fractionated by HSCCC using the two-phase solvent system heptane-acetonitrile-CH_2Cl_2 (10:7:3) in the descending mode with the lower phase as mobile phase, (flow rate: 2 mL/min, rotation: 500 rpm, volume of column: 1000 mL). 8-mL fractions were collected to obtain 21 fractions (F1 - F21).

The most polar fraction (F3, 529 mg, t_r between 180 and 240 mL) was purified by MPLC on silica gel 60 Merck® (15–25μM) eluted with toluene/EtOAc/HOAc (90:2:5), 6 mL/min, giving 7 fractions (F3.1 – F3.7). Fraction 3.7 (267.6 mg, t_r between 1858 and 2502 mL) was further purified by MPLC on silica gel 60 (15–25μM) eluted with toluene/EtOAc/HOAc (90:5:5) (flow rate: 6 mL/min) giving 3 fractions (F3.7.1 – F3.7.3). Fraction 3.7.2 (99 mg, t_r between 460 and 880 mL) was finally purified by MPLC on silica gel 60 (15–25 μM) eluted with toluene/EtOAc (93:7) (flow rate: 6 mL/min) to give 58 mg of a mixture of two compounds (t_r between 496 and 676 mL). The eluates were monitored by TLC (SiO_2 toluene/EtOAc/MeOH, 80:18:2, anisaldehyde-sulphuric acid reagent), R_f**1** and **2**:0.23. They were only separated by capillary GC on a DB-XLB column (J&W Scientific).

Isolation of **1** was only possible after hydroxylation of **2**: 10 mg of the mixture were dissolved in 1 mL of EtOAc/MeOH/H_2O (5:4:1) with 40 mg of *N*-methylmorpholine *N*-oxide and 600 μL of OsO_4 (0.1 M solution) at room temperature. The reaction was complete after 24 h.

Preparative TLC on silica gel (2 mm) Merck® without fluorescence indicator, using toluene/EtOAc/acetonitrile (50.20:30) as mobile phase allowed us to isolate 5 mg of compound **1** (R_f: 0.46) and 4 mg of hydroxylated pimarane (R_f: 0.08).

Cytotoxicity tests

Compounds were tested on HeLa cells as described by Block et al. [3]. The effect was evaluated using the tetrazolium salt MTT (Sigma) colorimetric method based on the cleavage of the reagent by dehydrogenase in viable cells. The relative absorbances were expressed as percent of the control and camptothecin (Sigma) was used as positive control.

Measurement of aorta contraction

Male Wistar rats weighing 200 to 300 g were sacrificed by decapitation. The descending thoracic aortas were isolated, cleaned and cut in rings (2 mm). Contractile responses were measured as described by El Bardai et al. [6]. Aortic rings were suspended under a resting tension of 20 mN in 12.5-mL organ baths filled with a physiological solution [composition (mM): NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; $MgCl_2$ 1.25; $CaCl_2$, 1.25; glucose, 11] maintained at 37 °C and bubbled with a gas mixture of 95% of O_2 and 5% CO_2.

Preparations were submitted to a basal tension of 20 mN and allowed to equilibrate for 60 min before initiating the experimental procedures. Contractions were induced by changing the physiological solution in the bath to a depolarising solution [100 mM; composition (mM): NaCl, 27; KCl, 100; $NaHCO_3$, 15; $MgCl_2$ 1.25; $CaCl_2$, 1.25; glucose, 11].

After washing, the muscles were pre-incubated for 30 min in the presence of the compounds and a second contraction was evoked in the continuous presence of the tested compounds. The amplitude of the contraction induced in the presence of the tested compounds was compared to the response in its absence. Tested compounds were dissolved in DMSO as stock solutions at 10 mg/mL. Due to their low water solubility, the highest concentration that could be realised in the organ bath was 3 μg/mL. Verapamil was dissolved in water.

Data were corrected for the change in contraction measured after aorta incubation with the same concentration of DMSO, which maximally decreased contraction by 5%.

Results and Discussion

Purification of the dichloromethane extract allowed us to isolate a mixture of two diterpenes having the same R_f and R_t values in TLC and HPLC systems and the same molecular formula, $C_{20}H_{32}O_2$.

Compound **1** {$[\alpha]_D^{22}$: −72° (CH_2Cl_2, *c* 0.1)} was isolated from the mixture as a white powder, whose molecular formula, $C_{20}H_{32}O_2$, was established by HR-EI-MS (*m/z* = 304.2402 $[M]^+$ calculated for $C_{20}H_{32}O_2$: 304.2402). Infrared absorptions at 3520 cm^{-1} provided evidence of hydroxy groups. The presence of a cyclopropane ring was deduced from the ^{1}H-NMR spectrum that showed two signals at δ_H = 0.57 and 0.82 ppm (H-12 and H-13, respectively), and by the ^{13}C-NMR spectrum that showed signals at δ_C = 20.5 (C-12), 24.2 (C-13), 22.5 (C-16) ppm. From these observations and comparisons with NMR data from closely related structures [3], [4], [7], [8], [9], [10], we could conclude that compound **1** belongs to the trachylobane series of diterpene. The NMR data are shown in Table **1**. The presence of two hydroxy groups was confirmed by the two signals at δ_C = 72.1 and 77.1 ppm in the ^{13}C-NMR spectrum.

Table 1 NMR data of compound **1** [$CDCl_3$, 500.200 MHz (^1H), 125.775 MHz (^{13}C), δ ppm (J in Hz)]

Position	^1H	^{13}C	HMBC (C→H)
1a	1.52 m	37.12	H-2, H-20
1b	0.86 m		
2	1.57 m	26.57	
3	3.61 dd (9.5, 7.0)	77.06	H-1a, H-19
4	–	41.81	H-2, H-18a, H-18b, H-19
5	0.84 m	49.69	H-18a, H18b, H-19, H-20
6	1.33 m	20.08	
7	1.38 m	38.60	H-15a
8	–	40.49	H-13, H-14a, H-15a, H15-b
9	1.09 dd (11.0, 7.3)	53.12	H-14a, H-15a, H15-b, H-20
10	–	37.89	H-2, H-19
11a	1.87 ddd (14.7, 11.0, 2.9)	19.70	
11b	1.66 ddd (14.7, 7.3, 2.6)		
12	0. 57 ddd (7.3, 2.9, 2.6)	20.51	H-15a, H-15b, H-17
13	0.82 m	24.21	H-11a, H-14a, H-15b, H-17
14a	2.04 d (12.0)	33.41	H-15b
14b	1.15 d (12.0)		
15a	1.39 d (11.3)	50.33	H-14a, H-17
15b	1.22 d (11.3)		
16	–	22.50	H-11b, H-14a, H-15a, H-15b, H-17
17	1.12 s	20.52	H-15a, H-15b
18a	3.69 d (10.3)	72.10	H-19
18b	3.39 d (10.3)		
19	0.87 s	11.26	
20	0.98 s	14.98	

The primary alcohol was revealed in the ^1H-NMR spectrum by the two doublets at 3.69 and 3.39 and the tertiary alcohol by the doublet of doublets at 3.61. These attributions were confirmed by the DEPT spectrum of the molecule. The stereochemistry was established by NOE correlations between H-18 (3.69 and 3.39), H-3 (3.61) and H-5 (0.84). The assignment of the tertiary alcohol at C-3 was confirmed by HMBC correlations between the protons at δ_H = 1.57 (H-2), 0.87 (Me-19), 3.69 (H-18a), 3.39 (H-18b) and the carbon at δ_C = 77.1 (C-3). The equatorial position of the hydroxy group at C-3 was deduced by the observation of the coupling constants of the dd at δ_H = 3.61 (J = 9.5 and 7.0 Hz, H-3) and by NOE correlations between H-3 (3.61) and H-5 (0.84). The full ^1H- and ^{13}C-NMR assignments were established with COSY, HMQC and HMBC correlations. All the naturally-occurring trachylobane diterpenes isolated so far belong to the *enantio* series, NOE correlations between H-5 and H-3, H-19 and H-20, H-17 and H-12, H-9 and H-12 and between H-5 and H-9 and comparison with other *ent*-trachylobanes confirmed the *ent*-configuration of compound **1** [3], [7], [8], [9], [10]. Compound **1** was identified as *ent*-18-hydroxytrachyloban-3β-ol and its structure was confirmed by X-ray crystallographic analysis (Figs. **1** and **2**).

Compound **2** could not be isolated with enough purity to undertake an NMR analysis. Its structure has been elucidated only by X-ray diffraction of a crystal of a mixture of the two compounds.

It has been identified as an *ent*-isopimarane diterpene: *ent*-18-hydroxyisopimara-7,15-dien-3β-ol (Fig. **1**). The absolute configuration could not be elucidated because of lack of NMR data but it could be deduced from X-ray diffraction knowing the absolute configuration of **1**.

Fig. **2** shows the ORTEP [11] view and atom labelling of *ent*-18-hydroxytrachyloban-3β-ol and of *ent*-18-hydroxyisopimara-7,15-dien-3β-ol .

X-Ray structure analysis

Colourless crystals were obtained by slow evaporation from a dichloromethane solution of the mixture of the 2 compounds. Molecular formula = $C_{20}H_{32}O_2$, M_r = 304.46, monoclinic, $P2_1$, a = 10.780(1), b = 7.373(1), c = 21.677(2) Å, β = 90.03(1)°, V = 1722.8(2) Å^3 , Z = 4, Dx = 1.17 g cm^{-3}, μ = 0.56 mm^{-1}, F(000) = 672 , T = 100 K.

A total of 14616 reflections were collected using a Bruker CCD detector and Cu-Kα radiation (λ = 1.54178 Å) – 6175 independent reflections (Rint = 0.081). The structure was solved by direct methods with SHELXS-97 [12] and refined by the least square using F^2 values and anisotropic thermal parameters for non-hydrogen atoms with SHELXL-97 [12]. The angle β was 90° but the orthorhombic symmetry was not observed. All the H atoms were calculated and included in the refinement with a common isotropic temperature factor. Final R values are: R 0.068 for 4867 observed reflections, R (all data) = 0.082, wR = 0.168, S = 0.99. The data have been registered with the Cambridge Crystallographic Data Centre (CCDC 240618).

Only four hydrogen bonds are observed in the structure. One of these four H-bonds is intramolecular, it is observed in molecule **1** between the two hydroxy groups forming a six-membered

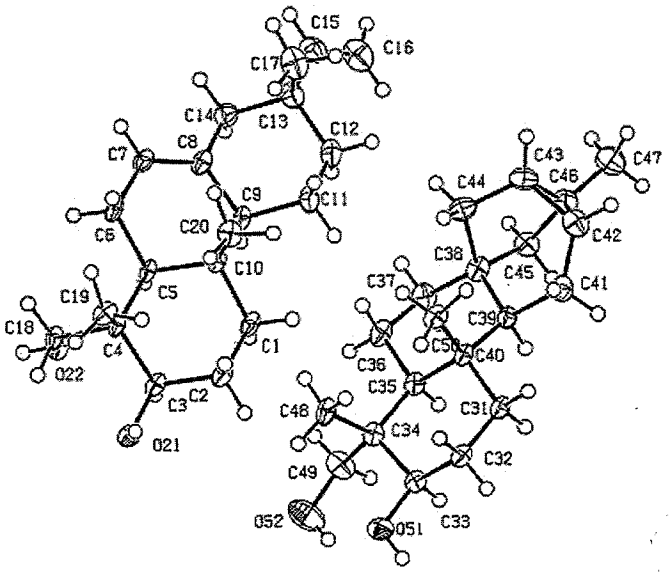

Fig. 2 ORTEP view and atom labelling of *ent*-18-hydroxytrachyloban-3β-ol and 18-hydroxyisopimara-7,15-dien-3β-ol.

ring. In **2**, the CH_2-OH group is nearly perpendicular to ring A of the tricyclic skeleton so that this intramolecular H-bond is no longer possible.

The three intermolecular H-bonds lead to an infinite two-dimensional network. It is noteworthy to mention that only one H-bond is observed between the two different entities **1** and **2**. It is probably this interaction between the two different molecules **1** and **2** that leads to the observed solid solution. Examples of such solid solutions have been found in steroids [13]. The X-ray structure of vitamin D1 reveals a sandwich-like heterodimer complex of very structurally dissimilar molecules [14].

As *Croton zambesicus* is used in African traditional medicine to treat hypertension, we investigated the activity of the mixture of **1** and **2**, of the isolated *ent*-18-hydroxytrachyloban-3β-ol and of the hydroxylated pimarane derivative on KCl-induced contraction. Marrubenol, a diterpene with known vasorelaxant activity associated with Ca^{++}-antagonist properties, and verapamil were used as reference compounds [6], [15].

The 1 : 1 mixture of the two compounds [0.3 – 3 μg/mL] did not affect aorta basal tone but inhibited the KCl-induced contraction in a concentration-dependent manner with an IC_{50} of 1 μg/mL (corresponding to 3.3 μM of diterpenes, both compounds having the same molecular weight: 304.24) while the IC_{50} values of marrubenol and verapamil were 3.3 and 0.057 μg/mL or 10 and 0.025 μM, respectively [6], [15]. The trachylobane and the hydroxylated pimarane showed a much lower activity than the 1 : 1 mixture which is about 3 times more active than marrubenol (Table **2**). As far as we know, trachylobane diterpenes are poorly known from a pharmacological point of view and no previous study on the vasorelaxant activity of trachylobanes has been reported. On the contrary, some pimaranes are known to possess vasorelaxant activity [16], [17].

In comparison to the weak activity of **1**, the higher activity of the mixture could be explained by a synergy between trachylobane and pimarane or, more probably, by a stronger activity of the pimarane component in its original non-hydroxylated form.

Table **2** Effects of *ent*-18-hydroxytrachyloban-3β-ol (**1**), the hydroxylated derivative of *ent*-18-hydroxyisopimara-7,15dien-3β-ol and a mixture of **1** and **2** on the contractions of rat aortic rings exposed to 100 mM KCl depolarizing solution

Compound	*Concentration (µg/mL)*	*% Residual contraction*
1 : 1 Mixture of compounds **1** and **2**	3	41.0 ± 6.1 (4)*
ent-18-Hydroxytrachyloban-3β-ol (**1**)	3	84.4 ± 6.1 (9)*
Hydroxylated *ent*-18-hydroxyisopimara-7,15-dien-3β-ol	3	93.9 ± 1.2 (4)*
Marrubenol	3	47.6 ± 3.2 (6)*
Verapamil	0.05	52.6 ± 6.7 (4)*
	0.5	15.8 ± 2.7 (4)*

Data (mean values of n determinations ± s. e. m.) are expressed as percentage of the contraction measured in the absence of drug (control). * $P < 0.05$ versus control.

Neither the mixture of the two compounds nor compound **1** exhibited cytotoxicity when tested on HeLa cells (IC_{50} >20 μg/mL).

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