ent-Trachyloban-3β-ol, a New Cytotoxic Diterpene from *Croton zambesicus*

S. Block[1], C. Stévigny[1], M.-C. De Pauw-Gillet[2], E. de Hoffmann[3], G. Llabrès[4], V. Adjakidjé[5], J. Quetin-Leclercq[1]

Abstract

The dichloromethane extract of leaves of *Croton zambesicus* (Euphorbiaceae) showing *in vitro* cytotoxicity against human cervix carcinoma cells was investigated in order to identify its active compounds. A bio-guided fractionation by HSCCC followed by MPLC led us to isolate a trachylobane diterpene, *ent*-trachyloban-3β-ol, with cytotoxic properties (IC_{50} on HeLa cells = 7.3 μg/ml) . This is the first report on the cytotoxicity of a trachylobane diterpene.

Croton zambesicus Muell. Arg. (Euphorbiaceae) (syn. *C. amabilis* Muell. Arg., syn. *C. gratissimus* Burch.) is a shrub or small tree reaching 10 m in height . It is a Guineo-Congolese species widespread in Tropical Africa [1]. The leaf decoction is used in Benin as anti-hypertensive and as anti-microbial (urinary infections) [1]. One previous phytochemical study of the stem bark of *C. zambesicus* has shown the presence of a new labdane diterpenoid (crotonadiol) with the known clerodane crotocorylifurane and two trachylobanes: 7β-acetoxytrachyloban-18-oic acid and trachyloban-7,18-diol [2]. We now report on the isolation from the leaves and characterisation of another trachylobane diterpene: *ent*-trachyloban-3β-ol (**1**) (Fig. **1**) with cytotoxic activity on HeLa cells. This compound was purified by a bioassay-guided fractionation of the crude dichloromethane extract of leaves by HSCCC followed by MPLC on silica gel.

Structural identification of **1** was carried out by analysing its physical and spectroscopic data. Compound **1** was isolated as a white powder (m. p. 165 – 167 °C). Its molecular weight was deduced on the basis of the EI mass spectrum which showed the

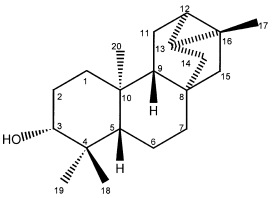

Fig. **1** Structure of *ent*-trachyloban-3β-ol (**1**).

Affiliation: [1] Laboratoire de Pharmacognosie, Unité d'Analyse Chimique et Physico-Chimique des Médicaments, Université Catholique de Louvain, UCL 72.30-CHAM, Bruxelles, Belgium · [2] CRCE, Histologie-Cytologie Université de Liège, Liège, Belgium · [3] Laboratoire de Spectrométrie de Masse, Université Catholique de Louvain, Louvain-la-Neuve, Belgium · [4] Département de Physique Expérimentale, Université de Liège, Liège, Belgium · [5] Département de Biologie Végétale, Faculté des Sciences et Techniques, Université Nationale du Bénin, Abomey-Calavy, Bénin

Correspondence: Sébastien Block · Laboratoire de pharmacognosie · Unité CHAM · UCL 72.30 · Avenue E. Mounier 72 · 1200 Bruxelles · Belgium · E-Mail: sebastien.block@cham.ucl.ac.be · Fax: +32 2 764.72.53

Received: October 22, 2001 · **Accepted:** February 24, 2002

Bibliography: Planta Med 2002; 68: 647–649 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943

$[M]^+$ peak at *m/z* 288 and peaks at *m/z* 273 $[M - CH_3]^+$, 270 $[M - H_2O]^+$ and 255 $[M - (CH_3 + H_2O)]^+$. Compound **1** showed no UV absorption. The ^1H- and ^{13}C-NMR values of (**1**) are consigned in Table **1**. Assignments were made on the basis of the ^1H, ^{13}C, HMQC and COSY spectra. Analysis of the ^1H-NMR spectrum revealed the presence of four methyl groups and two multiplets centered at δ_H 0.57 and at δ_H 0.81 corresponding to H-12 and H-13, respectively. This suggests a cyclopropane ring characteristic of the trachylobane diterpenes. The δ_C of C-12, C-13 and C-16 (respectively 20.61, 24.21 and 22.47) confirm the presence of the cyclopropane ring [3]. These ^{13}C- and ^1H-NMR spectra also show the absence of double bounds. The presence of the hydroxy group was deduced from the δ_C 79.24 (C-3) and from the δ_H 3.17 (H-3). The hydroxy substitution at position 3 was deduced on comparison with closely related natural compounds [3], [4], [5], [6], [7], [8] and analysis of correlations in the 2D spectra. As H-3 appeared as a *dd* (J = 10 and 5 Hz), the configuration of C-3 has been determined as axial [4], [5]. Comparison of the ^{13}C-NMR spectrum and the data obtained from the ^{13}C-NMR spectrum of *ent*-3β-acetoxy-trachyloban [8] already isolated from an acetylated fraction of the fruits of *Xylopia aromatica* confirms our assignments. The absolute configuration of **1** has not been determined but the compound is assumed to belong to the *enantio*-series of absolute configuration as all the other naturally occurring trachylobanes [3], [4], [5], [6], [7], [8].

Table **2** shows the cytotoxic activity on HeLa cells of the dichloromethane extract of the leaves of *Croton zambesicus* and of *ent*-trachyloban-3β-ol. This is the first report on the *in vitro* cytotoxic activities of *C. zambesicus* and of a trachylobane diterpene.

Table **1** ^1H- and ^{13}C-NMR data for compound **1** in $CDCl_3$

Carbon	*^{13}C chemical shifts*[a]	*Proton*	*^1H chemical shifts*[a]
1	38.68	1	1.32 (s) 1.41 (d)
2	26.92	2	1.55 (m)
3	79.24	3 ax	3.17 (dd) (J = 10 and 5 Hz)
4	39.00 [b]		–
5	55.11	5	0.72 (d)
6	20.53	6	1.30 (d)
7	38.01	7	1.54 (d) 0.85 (d)
8	40.49 [b]		–
9	53.16	9	1.06 (m)
10	38.68 [b]		–
11	20.46	11	1.65 (m) 1.86 (m)
12	20.61	12	0.57 (m)
13	24.21	13	0.81 (m)
14	33.20	14	2.03 (d) 1.16 (d)
15	50.36	15	1.22 (d) 1.43 (d)
16	22.47		–
17	20.61	17	1.12 (s)
18	28.06	18	0.95 (s)
19	15.44	19	0.75 (s)
20	14.62	20	0.92 (s)

[a] Chemical shifts (δ) in ppm. Multiplicities are in parentheses.
[b] These values can be interchanged.

Table **2** Cytotoxic activity on HeLa cells of the dichloromethane extract of leaves of *C. zambesicus* and of compound (**1**). Camptothecin was used as cytotoxic reference compound

Extracts and compound	*IC_{50} (µg/ml)*
Dichloromethane extract of the leaves of *C. zambesicus*	36.2
ent-trachyloban-3β-ol	7.3
Camptothecin	0.01

Materials and Methods

General experimental procedures: 1D and 2D spectra were recorded on a Bruker Avance DRX-400 spectrometer in $CDCl_3$ at 400.132 MHz (^1H) and 100.62 MHz (^{13}C), at 25 °C. Standard Bruker pulse sequences were used for homonuclear (COSY) and heteronuclear (HMQC) correlation experiments. MS analyses were achieved using electronic impact ionisation (EIMS, 70 eV) on a Finnigan TSQ700 triple quadrupole. Optical rotations were measured with a Perkin-Elmer 241 spectropolarimeter in $CHCl_3$ solution.

Plant material: The aerial parts of *C. zambesicus* were collected in Cotonou area (Benin) and identified by Prof. V. Adjakidje (Université Nationale du Bénin). A voucher has been deposited at the herbarium of the Belgian national botanical garden at Meise (BR S.P. 848.108).

Extraction and isolation: The air dried leaves (580 g) were powdered, macerated (1.8 l) and percolated (3.2 l) at room temperature with dichloromethane. Removal of the solvent under reduced pressure in a rotary evaporator yielded a dark green extract (34 g).

Part of this extract (1 g) was subjected to purification by high speed counter-current chromatography (HSCCC, Kromaton III SEAB) using the two phase solvent system heptane-acetonitrile-dichloromethane (10 : 7 : 3) (descending mode, mobile phase: lower phase, flow rate: 2 ml/min, fraction collection: 4 min/tube, rotation: 500 rpm, volume of column: 1000 ml). The eluates were monitored by TLC on silica gel 60 F_{254} Merck with toluene-ethyl acetate-methanol (80 : 18 : 2) as mobile phase. Anisaldehyde-sulfuric acid reagent was used for the detection. After TLC analysis, the fractions with similar chromatographic profiles were combined and tested for their cytotoxicity. The most active fraction (Fraction 20, 50 mg, IC_{50} = 13.7 µg/ml) was purified by MPLC on silica gel (Lichroprep Si 60 Merck, omnifit glass column OM 6427 15 × 750 mm) using toluene-ethyl acetate (90 : 10) as mobile phase (flow rate: 1 ml/min, fraction collection : 8 min/tube). Fraction 5 contained 3.5 mg of pure *ent*-trachylobane-3β-ol (**1**) ($[\alpha]_D^{22}$: –57° (*c* 0.1, $CHCl_3$)). Others fractions not containing **1** showed low cytotoxic activities (IC_{50} > 75 µg/ml) and were not further purified.

Cytotoxicity assay: HeLa cells were grown in minimum Eagle"s medium (MEM, Gibco) supplemented with 10% heat-inactivated foetal bovine serum and penicillin (100U/ml). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Stock solutions were prepared at 10 mg/ml in DMSO. The effect of the

extract and the different fractions of *C. zambesicus* was evaluated using the tetrazolium salt MTT (Sigma) colorimetric method based on the cleavage of the reagent by mitochondrial dehydrogenase in viable cells. Briefly, 5000 cells/well were seeded in 200 μl of medium in 96-well microculture plates for 24 h. After 24 h the medium was replaced by 200 μl of fresh medium containing various drug concentrations while control cells received fresh medium containing analogous DMSO concentrations. Each concentration was tested in at least 6 wells. After 72 h incubation, the medium was replaced by 100 μl DMEM (without serum) containing 10 μl of MTT solution (3 mg/ml in PBS). After 45 min in the incubator, the medium was removed and 100 μl of DMSO were added to each well. The plates were shacked and absorbances were recorded at two wavelength (570 nm and 620 nm), against a background control as blank (DMEM plus 10 μl of MTT solution in the absence of cells). Camptothecin (Sigma) was used as positive control [9]. The relative absorbances were expressed as percent of the control (100% in ordinate). IC_{50} determination was achieved via regression analysis of the results at three different concentrations of each drug. All experiments were made at least in duplicate.

Acknowledgements

The authors wish to thank R. Rozenberg and M. C. Fayt for their skillfull technical assistance. We also thank Professor J. Hanuise for the optical rotation measurement. This work was supported by a grant from the "fonds spécial de recherche" of the Catholic University of Louvain and the Belgian National Fund for Scientific Research (Funds no. 1.5.115.00).

References

[1] Adjanohoun EJ, Adjakidje V, de Souza S. Contribution aux études ethnobotaniques et floristiques en République Populaire du Bénin, Vol. 1: p 245 1989 Agence de coopération culturelle et technique,
[2] Ngadjui BT, Folefoc GG, Keumedjio F, Dongo E, Sondengam BL, Connolly JD. Crotonadiol, a labdane diterpenoid from the stem bark of *Croton zambesicus*. Phytochemistry 1999; 51: 171 – 4
[3] Arnone A, Mondelli R. ^{13}C-NMR spectroscopy of natural substances IV – ^{13}C-NMR studies of trachylobane diterpenes: complete carbon assignment. Organic Magnetic Resonance 1979; 12: 429 – 31
[4] Kapingu MC, Guillaume D, Mbwambo ZH, Moshi MJ, Uliso FC, Mahunnah RLA. Diterpenoids from the roots of *Croton macrostachys*. Phytochemistry 2000; 54: 767 – 70
[5] Leong YW, Harrison LJ. *Ent*-Trachylobane diterpenoids from the liverwort *Mastigophora diclados*. Phytochemistry 1997; 45: 1457 – 9
[6] Fraga BM. The trachylobane diterpenes. Phytochemical analysis 1994; 5: 49 – 56
[7] Harrigan GG, da S.Bolzani V, Gunatilaka AAL, Kingston DGI. Kaurane and trachylobane diterpenes from *Xylopia aethiopica*. Phytochemistry 1994; 36: 109 – 13
[8] Moraes MPL, Roque NF. Diterpenes from the fruit of *Xylopia aromatica*. Phytochemistry 1988; 27: 3205 – 8
[9] Huang R-L, Chen C-C, Huang Y-L, Ou J-C, Hu C-P, Chen C-F, Chang C. Anti-tumor effects of *d*-dicentrine from the root of *Lindera megaphylla*. Planta Medica 1998; 64: 212 – 5