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Diterpenes from the leaves of *Croton zambesicus*

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Abstract

Two new trachylobane- and one isopimarane-type diterpenoids: *ent*-18-hydroxy-trachyloban-3-one; *ent*-trachyloban-3-one; isopimara-7,15-dien-3β-ol, were isolated from the leaves of *Croton zambesicus*, together with *trans*-phytol, β-sitosterol, α-amyrin and stigmasterol. The structures were determined by extensive NMR techniques and X-ray analysis. The cytotoxicity of these compounds has been evaluated on cancer and non-cancer cell-lines.

Keywords: *Croton zambesicus*; Euphorbiaceae; Diterpene; Trachylobane; Pimarane; Cytotoxicity

1. Introduction

Croton zambezicus Muell. Arg. (Euphorbiaceae) (Syn. *C. amabilis* Muell. Arg., *C. gratissimus* Burch.) is a shrub or small tree reaching 10 m in height. It's a Guineo-Congolese species widespread in Tropical Africa (Adjanohoun et al., 1989). The leaf decoction is used in Benin as anti-hypertensive, anti-microbial (urinary infections) and to treat fever associated with malaria (Adjanohoun et al., 1989; Watt and Breyer-Brandwikj, 1962). The genus *Croton* is well known for its diterpenoid content and a lot of different types of diterpenes (phorbol esters, clerodane, labdane, kaurane, trachylobane, pimarane, etc.) have been isolated from this genus.

There is very little literature concerning the phytochemical study of *Croton zambesicus* although if this plant is widely used in African traditional medicine. Labdane, clerodane and trachylobane diterpenes have been identified in the stem bark of *Croton zambesicus* (Ngadjui et al., 2002). Recently we have identified a new cytotoxic trachylobane diterpene from the leaves of *C. zambesicus* (Block et al., 2002). In order to continue our investigations on the composition of the cytotoxic dichloromethane extract of the leaves we have isolated and characterised two new trachylobane and one isopimarane diterpenes together with *trans*-phytol, α-amyrin and sterols.

2. Results and discussion

HSCCC separation of the dichloromethane extract from the leaves of *C. zambesicus* gave 21 fractions. These fractions were further purified by MPLC. From these fractionations, we isolated five diterpenes: *ent*-trachyloban-3β-ol (Block et al., 2002), *ent*-18-hydroxy-trachyloban-3-one (**1**), isopimara-7,15-dien-3β-ol (**2**), *ent*-trachyloban-3-one (**3**) and *trans*-phytol (**4**) together with α-amyrin and sterols: β-sitosterol and stigmasterol. All these compounds were isolated for the first time from the leaves of *C. zambesicus*.

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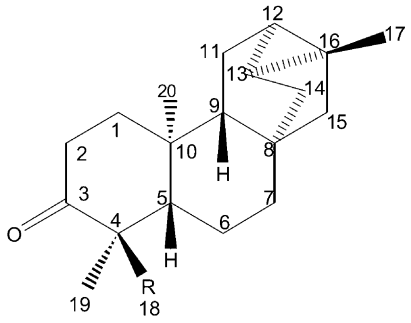

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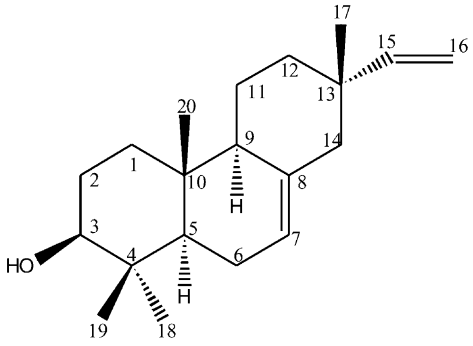

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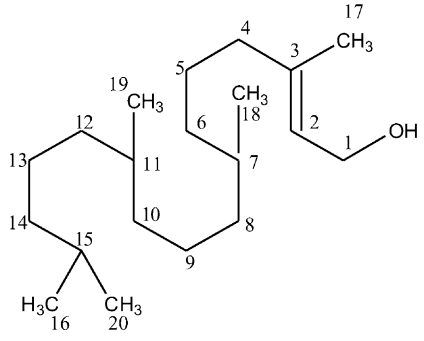

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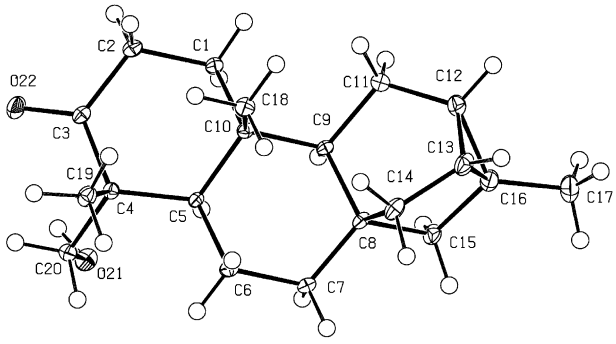

Fig. 1. View and atom labelling of one molecule from the asymmetric unit of **1** (Spek, 1998).

Compound **1** was isolated as a white crystal, whose molecular formula, $C_{20}H_{30}O_2$, was established by HR-EIMS. Infrared absorptions at 3520 and 1702 cm^{-1} provided evidence of respectively hydroxyl and carbonyl groups. The presence of a cyclopropane ring was deduced from the ^1H NMR spectrum that exhibits two signals at δ_H 0.62 and 0.87 ppm (H-12 and H-13 respectively) and by the ^{13}C NMR spectrum that shows signals at δ_C 20.4 (C-12), 24.2 (C-13), 22.5 (C-16) ppm. From these observations and comparison with NMR data from closely related structures (Block et al., 2002; Midiwo et al., 1997; Hasan et al., 1982; Arnone et al., 1979; Leong et al., 1997) we could conclude that compound **1** belongs to the trachylobane series of diterpene.

The presence of the carbonyl group was confirmed by the signal at δ_C 219.1 ppm in the ^{13}C spectrum. The primary alcohol was revealed by the two doublets at δ_H 3.63 and 3.37 ppm in the ^1H spectrum and by the signal at δ_C 66.8 ppm in the ^{13}C spectrum (Fig. 1).

Long range ^1H-^{13}C correlations (HMBC) between the three protons at δ_H 0.99 (Me-19) and the ^{13}C NMR signal at δ_C 66.8 and X-ray analysis supported the C-18 position for the hydroxyl group. The position of the ketone at C-3 was also deduced from HMBC correlations between the protons at δ_H 2.22 (H-2α), 2.62 (H-2β), 0.99 (Me-19), 3.63 (H-18a), 3.37 (H-18b) and the ketonic carbon at δ_C 219.1. The full ^1H and ^{13}C NMR assignments were established with HMQC correlations. X-ray crystallographic analysis was conducted to confirm the structure of **1**. All the naturally-occurring trachylobane diterpenes isolated so far belong to the *enantio* series and comparison with other *ent*-trachylobanes confirms the *ent*-configuration of **1** (Block et al., 2002; Midiwo et al., 1997; Hasan et al., 1982; Arnone et al., 1979; Leong et al., 1997). **1** is then identified as *ent*-18-hydroxy-trachyloban-3-one.

Compound **2** was isolated as a white solid with a molecular composition of $C_{20}H_{32}O$ as inferred from HR-EIMS. The IR spectrum showed absorption bands for a hydroxyl group (3314 cm^{-1}) and for a mono-substituted double bond (3099, 1639, 909 cm^{-1}). The combined analysis of the ^{13}C NMR and DEPT spectra revealed the presence of 20 carbon signals assigned to four methyls, seven methylenes, five methine among which one tertiary alcohol and two olefinic carbons and four quaternary carbons. The occurrence in the ^1H spectrum of three *dd* at δ_H 5.80 ($J = 10.8$ and 17.6 Hz, H-15), at δ_H 4.93 ($J = 1.6$ and 17.6 Hz, H-16B) and at δ_H 4.87 ($J = 1.6$ and 10.8 Hz, H-16A) associated with the presence of a broad doublet at δ_H 5.37 ($J = 3.5$ Hz, H-7) and four singlets corresponding to methyl groups suggested a pimarane type skeleton (Lago et al., 2000). The position of the alcohol on the skeleton of the pimarane was determined as C-3 by the HMBC correlation between the proton at δ_H 3.26 (H-3) and the ^{13}C NMR signals δ_C 27.4 (C-2) and 37.8 (C-4) and by the ^1H–^1H

COSY correlation between the protons δ_H 3.26 (H-3) and 1.62 (H-2). The equatorial position of the hydroxyl group at C-3 was deduced by the observation of the coupling constants of the *dd* at 3.26 ($J = 4.7$ and 10.9 Hz, H-3α). The position of the double bound between C-7 and C-8 was defined by the ^{1}H-^{1}H COSY correlation between the protons δ_H 5.37 (H-7) and δ_H 1.97 (H-6). The full ^{1}H and ^{13}C NMR assignments were established with HMQC correlations. The stereochemistry at C-13 was established by comparison of the ^{13}C NMR chemical shifts of C-15, C-16 and C-17 with those of isopimarane diterpenoids, showing an equatorial position for the Me-17 and an axial position for the vinyl group (Beier, 1978; Wenkert and Buckwalter, 1972; Rasoamiaranjanahary et al., 2003; Polonsky et al., 1970; Anjaneyulu et al., 2003; Lago et al., 2000). Compound **2** was finally identified as isopimara-7,15-dien-3β-ol. This compound has already been synthesised from virescenol A (Polonsky et al., 1970; Ceccherelli et al., 1985) and isolated from the leaves of *Guarea macrophylla* (Lago et al., 2000) but this is the first pimarane-type diterpene isolated from *C. zambesicus*. Moreover, comparison of NMR data of **2** with those reported by Lago (Lago et al., 2000) shows very good agreement excepted for the chemical shifts of C-2 (27.4) and C-6 (23.1) that are inverted. Our assignments were confirmed by the HMBC correlation between the proton at δ_H 3.26 (H-3) and the ^{13}C NMR signal δ_C 27.4 (C-2) and by the ^{1}H–^{1}H COSY correlations between the protons δ_H 3.26 (H-3) and 1.62 (H-2) and between the protons δ_H 5.37 (H-7) and δ_H 1.97 (H-6) and by comparison with closely related structure (Ansell et al., 1993; Meragelman et al., 2003; Aiyar et al., 1969, 1971). Complete ^{13}C and ^{1}H NMR assignments of **2** are presented in Table 2.

Compound **3** was isolated as a colourless oil. Its molecular formula was determined as $C_{20}H_{30}O$ by HR-EIMS analysis. Compound **3** contained a carbonyl group as inferred from the IR absorption band at 1706 cm^{-1}. The trachylobane skeleton of this compound was, as compound **1**, identified by the presence of the cyclopropane ring signals in the ^{1}H (δ_H 0.61 and 0.85 ppm respectively for H-12 and H-13) and ^{13}C (δ_C 20.5, 24.2 and 22.5 ppm respectively for C-12, C-13 and C-16)

Table 1
^{13}C and ^{1}H NMR spectroscopic data for diterpenes **1** and **3** in $CDCl_3$. ^{13}C NMR at 125 MHz for **1** and 100 MHz for **3.** ^{1}H NMR at 500 MHz for **1** and 400 MHz for **3**. Chemical shifts are given in ppm; multiplicities and coupling constant *J* (in parentheses) in Hz

Position	**1**		**3**	
	δ_C	δ_H	δ_C	δ_H
1α	38	1.46 *m*	38	1.44 *m*
1β		1.46 *m*		1.72 *m*
2α	35	2.22 *m*	34.1	2.30 *m*
2β		2.62 *m*		2.55 *m*
3	219.1	–	217.5	–
4	52.5	–	47.6	–
5α	52.3	1.21 *m*	55.5	1.24 *m*
6α	20.6	1.43 *m*	21.1	1.41 *m*
6β		1.43 *m*		1.41 *m*
7α	37.8	1.25 *m*	38.3	1.24 *m*
7β		1.83 *m*		1.80 *m*
8	40.4	–	40.4	–
9α	49.2	1.59 *m*	52.4	1.22 *m*
10	37.5	–	37.7	–
11α	19.7	1.94 *m*	19.6	1.93 *m*
11β		1.72 *m*		1.74 *m*
12α	20.4	0.62 *d* (7.8)	20.5	0.61 *d* (7.6)
13α	24.2	0.87 *dd* (3.1, 7.8)	24.2	0.85 *dd* (3.2, 7.6)
14α	33.5	1.24 *m*	33.2	1.23 *m*
14β		2.09 *d* (11.9)	2.07 *d* (12.0)	
15α	50.2	1.44 *d*	50.2	1.44 *d*
15β		1.27 *d*		1.26 *d*
16	22.5	–	22.5	–
17	20.5	1.14 *s*	20.4	1.13 *s*
18a	66.8	3.63 *d* (11.4)	26	1.05 *s*
18b		3.37 *d* (11.4)		
19	16.8	0.99 *s*	21.5	1.01 *s*
20	14.4	1.18 *s*	14.1	1.10 *s*

Table 2
NMR assignments of compound **2** in $CDCl_3$ (^{13}C at 100 MHz and ^{1}H at 400 MHz). Chemical shifts are given in ppm; multiplicities and coupling constant *J* (in parentheses) in Hz

Position	**2**	
	δ_C	δ_H
1α	38.6	1.25 *m*
1β		1.84 *m*
2α	27.4	1.62 *m*
2β		1.62 *m*
3α	79.3	3.26 *dd* (4.7, 10.9)
4	37.8	–
5α	50	1.16 *dd* (4.9, 11.9)
6α	23.1	1.97 *m*
6β		1.97 *m*
7	121.4	5.37 *bd* (3.5)
8	135.4	–
9α	51.9	1.63 *m*
10	37.3	–
11α	20.1	1.57 *m*
11β		1.39 *m*
12α	36.1	1.36 *m*
12β		1.53 *m*
13	35.3	–
14α	45.9	1.95 *m*
14β		1.95 *m*
15	150.3	5.80 *dd* (10.8, 17.6)
16A	109.2	4.87 *dd* (1.6, 10.8)
		4.93 *dd* (1.6, 17.6)
16B		
17	21.4	0.86 *s*
18	28.3	1.00 *s*
19	15.6	0.90 *s*
20	14.9	0.87 *s*

NMR spectra. The presence of the carbonyl was deduced from the signal at δ_C 217.5 ppm on the ^{13}C spectrum and the position on C-3 was deduced from HMBC spectra showing correlation between the protons at δ_H 2.55 (H-2β) and 1.05 (H-18) and the carbon at δ_C 217.5. The stereochemistry of **3** was based on biosynthetic considerations (all natural trachylobanes isolated up to now belong to the *enantio* series) and on comparison of spectral data from **1** and closely related compounds (Kapingu et al., 2000; Ngouela et al., 1998; Arnone et al., 1979; Leong et al., 1997). **3** is then identified as *ent*-trachyloban-3-one. In order to complete the study on the cytotoxic activity of the dichloromethane extract of *C. zambesicus*, the isolated diterpenes, α-amyrin and sterols were tested in vitro against cancer (HeLa, HL-60) and non-cancer (WI-38) cell lines. The results are presented in Table 3.

The biological activities of trachylobane diterpenes are poorly known but recently we have shown that *ent*-trachyloban-3β-ol possesses cytotoxic activities on HeLa cells (IC_{50} on HeLa cells = 7.3 μg/ml). The cytotoxicities of compounds **1** and **3** are a little bit lower but no clear specificity between cell lines could be observed even if **3** is 2.5 more active on HeLa cells (cancer cell line) than on WI-38 (non-cancer cell line).

Different biological properties have been described for various pimarane derivatives, including antimicrobial and spasmolytic (Vlietinck, 1987), antihypertensive (Ohashi et al., 2000), antituberculosis (Ulubelen et al., 1997), antifungal (Rasoamiaranjanahary et al., 2003) and antiinflammatory described as international patent (Suh et al., 1999). Studies have also demonstrated that pimarane derivatives inhibited the tumor-promoting effect of TPA (12-*O*-tetradecanoylphorbol 13-acetate) and were slightly cytotoxic (Minami et al., 2002; Chang et al., 2000) suggesting an interesting cancer chemopreventive potential. The results obtained on the cytotoxicity of compound **2** confirm the weak cytotoxic activity of pimarane diterpenes. In comparison to the other diterpenes, *trans*-phytol (**4**) shows a similar range of activity than trachylobanes. The cytotoxic activity of phytol is due to an induction of apoptosis (Komiya et al., 1999). Finally, in agreement with literature data (Chaturvedula et al., 2002; Awad et al., 2000; Moghadasian, 2000), β-sitosterol, α-amyrin and stigmasterol were not cytotoxic at the tested concentrations (IC_{50} > 30 μg/ml on every cell lines).

Table 3
Cytotoxicity data for compounds **1**–**4**[a]

Compound	Cell lines[b]		
	HeLa	HL-60	WI-38
1	12.2±2.1	12.7±1.2	18.3±2.7
2	25.3±3.3	28.9±4.0	32.6±3.6
3	9.6±1.6	12.4±1.9	23.8±3.2
4	13.8±1.3	16.4±2.0	13.8±1.7
Camptothecin	0.5 μM	0.1 μM	0.6 μM

[a] Results are expressed as mean of IC_{50} values (μg/ml)±SEM of three independent experiments.

[b] HeLa, human cervix carcinoma; HL-60, human promyelocytic leukemia; WI-38, non-cancer human lung fibroblast.

3. Experimental

3.1. General

High Speed Counter-Current Chromatography was performed on a 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 sulfuric anisaldehyde, followed by heating 5 min at 105 °C. The IR spectra were recorded on a Perkin Elmer FTIR 286. The optical rotation values were obtained on a Perkin-Elmer 241 spectropolarimeter in CH_2Cl_2 solution. UV spectra were measured on a Uvikon 933 (Kontron) spectrophotometer. NMR spectra of compounds **2** and **3** were recorded on a Bruker Avance DRX-400 spectrometer in $CDCl_3$ at 400 MHz (^1H) and 100 MHz (^{13}C), at 25 °C. NMR spectra of compound **1** were recorded on a Bruker Avance 500 at 500 MHz (^1H) and 125 MHz (^{13}C); δ in ppm rel. to Me_4Si (internal standard). HR-EIMS was recorded at 70 eV in an AutoSpec 6 F mass spectrometer and EIMS at 70 eV on a Finnigan TSQ7000 triple quadrupole; m/z (rel. intensity in%).

3.2. Plant material

The aerial parts of *C. zambesicus* were collected in the surroundings of Cotonou (Benin) in December 2000 and identified by botanist Prof. V. Adjakidje (Université d'Abomey-Calavi-Benin). A voucher specimen has been deposited at the herbarium of the Belgian national botanical garden at Meise (BR S.P. 848.108).

3.3. Extraction and isolation

Air-dried and powdered leaves (580 g) were percolated at room temperature with dichloromethane to give 34 g of extract. Part of this extract (5 g) was fractionated by HSCCC using the two phases 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). 21 fractions (F1–F21) were obtained. F6 (315 mg) was separated by MPLC on silicagel 60 (15–25 μM) eluted with Tol–CH_3CN (93:7) giving 6 fractions (F61–F66). Fraction

F65 (59.4 mg) was finally purified by MPLC on silicagel 60 (15–25 μM) eluted with Tol–EtOAc–CH_3CN (91:8:1) to give compound **1** (20 mg). Fraction F9 (350.6 mg) was separated by MPLC on silicagel 60 (15–25 μM) eluted with Tol–EtOAc (98:2) giving 8 fractions (F91–F98). Fraction F94 (49.5 mg) was purified by MPLC on silicagel 60 (15–25 μM) with Tol–EtOAc (96:4) as mobile phase to afford compound **2** (14 mg). Fraction F12 contained *ent*-trachyloban-3β-ol, previously identified in the plant (Block et al., 2002). Fraction F14 (256 mg) was applied to MPLC on silicagel 60 (15–25 μM) eluted with Tol–EtOAc (93:8). 7 fractions (F141–F147) were obtained. Fraction F142 was purified by MPLC on silicagel 60 (15–25 μM), using Tol–EtOAc (90:10) as mobile phase to give trans-phytol (**4**) (8 mg). Fraction F144 was purified by MPLC on silicagel 60 (15–25 μM), Tol–EtOAc (92:8) was used as mobile phase and 25 mg of compound **3** were obtained. F17 gave α-amyrin and β-sitosterol. F18 gave stigmasterol.

3.4. *ent*-18-Hydroxy-trachyloban-3-one (*1*)

White crystals [CH_2Cl_2]. $[\alpha]_D^{22}$: −77° (CH_2Cl_2, *c* 0.1); UV λ_{max} nm (log ϵ): 218 (2.23); IR ν_{max}^{NaCl} cm^{-1}: 3520 (OH), 2988, 2928, 2859, 1702 (C=O), 1460, 1444, 1417, 1380, 1256, 1209, 1164, 1094, 1082, 1047, 1011, 975, 844, 757; ^1H and ^{13}C are given in Tables 1; EI-MS 70 eV *m*/*z* (rel. int.): 302 $[M]^{+\cdot}$ (30), 284 $[M-H_2O]^+$ (45), 272 (48), 269 (26), 257 (16), 246 (7), 215 (6), 201(5), 187 (4), 185 (2), 159 (1), 145 (1), 107 (3), 105 (17), 93 (22), 91 (42), 81 (36), 79 (83), 55 (100). HR-EIMS *m*/*z*: 302.2249 $[M]^{+\cdot}$ (calc. for $C_{20}H_{30}O_2$ 302.2246).

3.5. Isopimara-7,15-dien-3β-ol (*2*)

Amorphous powder. $[\alpha]_D^{22}$: +15° (CH_2Cl_2, *c* 0.1); UV λ_{max} nm (log ϵ): 226 (2.61); IR ν_{max}^{NaCl} cm^{-1}: 3314 (OH), 2953, 2926, 2968, 1669, 1463, 1378, 1366, 1002; ^1H and ^{13}C are given in Table 2; EI-MS 70 eV *m*/*z* (rel. int.): 288 $[M]^{+\cdot}$ (5), 273 $[M-CH_3]^+$ (8), 270 $[M-H_2O]^+$ (4), 255 (26), 245 (9), 227 (7), 213 (9), 200 (17), 185 (19), 171 (19), 145 (44), 134 (50), 132 (66), 131 (99), 129 (100), 119 (87), 105 (69), 91 (24). HR-EIMS *m*/*z*: 288.2448 $[M]^{+\cdot}$ (calc. for $C_{20}H_{32}O$ 288.2453).

3.6. *ent*-Trachyloban-3-one (*3*)

Colorless oil. $[\alpha]_D^{22}$: −37° (CH_2Cl_2, *c* 0.1); UV λ_{max} nm (log ϵ): 228 (2.78); IR ν_{max}^{NaCl} cm^{-1}: 2969, 2933, 2860, 1706 (C=O), 1458, 1384, 1367, 1261, 1202, 1112, 1082, 1010, 844; ^1H and ^{13}C are given in Table 1; EI-MS 70 eV *m*/*z* (rel. int.): 286 $[M]^{+\cdot}$ (77), 271 $[M-CH_3]^+$ (17), 253 (1), 230 (23), 215 (12), 200 (11), 173 (5), 159 (8), 145 (12), 131 (8), 119 (14), 107 (11), 105 (100), 93 (16), 91 (15), 81 (8), 79 (10), 55 (9). HR-EIMS *m*/*z*: 286.2293 $[M]^{+\cdot}$ (calc. for $C_{20}H_{30}O$ 286.2296).

3.7. X-Ray structure analysis of compound *1*

Colourless crystals were obtained by slow evaporation from a dichloromethane solution. $C_{20}H_{30}O_2$, Mr = 302.44, monoclinic, space group P 2_1, $a = 7.311(1)$, $b = 42.210(1)$, $c = 10.903(1)$ Å, $\beta = 91.10(1)°$, $V = 3363.8(1)$ $Å^3$, $Z = 8$, $Dx = 1.20$ g cm^{-3}, $\mu = 0.577$ mm^{-1}, $F(000) = 1328$, $T = 120$ K.

A total of 29,725 reflections were collected using a Bruker SMART 6000 CCD detector and CuK_α radiation ($\lambda = 1.54178$ Å). 6532 independent refection ($R_{int} = 0.052$). The structure was solved by direct methods with SHELXS-97 (Sheldrick, 1997) and refined by least-squares using F^2 values and anisotropic thermal parameters for non-hydrogen atoms with SHELXL-97 (Sheldrick, 1997). The H atoms of the hydroxyl groups were localized from difference Fourier maps; all the other H atoms were calculated and included in the refinement with a common isotropic temperature factor. Final *R* values are: $R = 0.041$ for 6370 observed reflections, *R* (all data) = 0.042, $wR_2 = 0.109$, $S = 1.02$, Flack parameter = 0.08(14). The data have been deposit with the Cambridge Crystallographic Data Centre (Nr CCDC 222992).

The four independent molecules are similar except that the conformation of ring I (C1–C2–C3–C4–C5–C10) is clearly a less flattened chair in molecule 3 (labelled C301–C310) than in the three other ones (in molecule 3, the endocyclic torsion angles are : −52, 47, −43, 48, −54 and 54° while the mean values for molecules 1 2 and 4 are −50, 34, −29, 40, −54 and 58°). Selected average bond lengths are (Å): C(3)–O(22) = 1.220(3), C(20)–O(21) = 1.425(3). The four OH groups are hydrogen-bonded to a C=O of another molecule making infinite one-dimensional chains.

3.8. Cytotoxicity assay

HeLa (human cervix carcinoma cells) and WI-38 (human lung fibroblast) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum (Gibco BRL) and antibiotics (100 IU penicillin/ml, 100 μg streptomycin/ml). HL-60 (human promyelocytic leukemia) cells were routinely grown in suspension in RPMI 1640 medium (Gibco BRL) containing 0.33% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, antibiotics (100 IU penicillin/ml, 100 μg streptomycin/ml) and supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Stock solutions of compounds were prepared at 10 mg/ml in DMSO and stored at 4 °C. The cytotoxicity of the compounds on HeLa and WI-38 cells was evaluated using the tetrazolium salt MTT (Sigma) colorimetric method based on the cleavage of the reagent by dehydrogenases in viable

cells. Briefly, 5000 HeLa or WI-38 cells per well were seeded in 100 μl of DMEM in 96-well microculture plates for 24 h. After 24 h adaptation, 100 μl of medium containing various drug concentrations were added to each well, while control cells received fresh medium containing analogous DMSO concentrations. Each concentration was tested in at least 8 wells. After 72 h incubation, the medium was replaced by 100 μl DMEM (without serum) medium 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 shaked and optical densities were recorded at two wavelengths (570 nm and 620 nm), against a background control as blank (100 μl of pure DMSO). The cytotoxicity on HL-60 cells was evaluated using another tetrazolium salt, WST-1 (Boehringer). Briefly, 50000 HL-60 cells in 100 μl of RPMI 1640 medium were seeded in each well of a 96-well plate. 100 μl of fresh medium containing various drug concentrations were added to each well while control cells received fresh medium with analogous concentrations of DMSO. Each concentration was tested in at least 8 wells. After 72 h treatment, each well was supplemented with 10 μl of WST-1 and then incubated for 45 min. Afterwards the plates were shaken and the optical density was measured at 450 and 620 nm against a background control as blank on a microplate reader. For the 3 cell lines, the relative optical density was expressed as percent of the control cells considered as 100%. In each case, camptothecin (Sigma) was used as positive control. IC_{50} determination was achieved via regression analysis of the results of at least 5 different concentrations of each drug. Results are mean ± SEM of 3 independent experiments.

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