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Phytochemistry 66 (2005) 627–632
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Cladocalol, a pentacyclic 28-*nor*-triterpene from *Eucalyptus cladocalyx* with cytotoxic activity

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Received 11 November 2004; received in revised form 13 December 2004
Available online 25 January 2005

Dedicated to the memory of Professor Antonio G. González

Abstract

A formylated triterpene named cladocalol has been isolated from the leaves of *Eucalyptus cladocalyx*, together with ursulolactone acetate, ursolic acid, 3β-acetate-12,20(29)-lupadien-28-oic acid, β-sitosterol and the known flavonoid eucalyptine. Their structures were mainly established by extensive NMR studies (^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H–^{1}H COSY, HSQC, HMBC) and mass spectroscopy as well as by X-ray crystallographic analysis. In this paper, we report on the cytotoxic effect induced by cladocalol and its derivatives on the myeloid leukemia cell line HL-60.

Keywords: *Eucalyptus cladocalyx*; Myrtaceae; Pentacyclic triterpenes; Sterol; Flavonoid; Single-crystal X-ray analysis; Cytotoxic

1. Introduction

The genus *Eucalyptus* (Myrtaceae) is native to Australia (Penford and Willis, 1961) and it is cultivated for its economic interest mainly in the paper industry (Eldridge et al., 1993), and for pharmaceuticals and cosmetics (Coppen, 2002). The leaves of several species are used for the production of essential oils with analgesic, expectorant, antiinflammatory and antimicrobial properties (Silva et al., 2003; Williams et al., 1998).

As a part of our continuing phytochemical investigation of Algerian plants (Medjroubi et al., 2003; Benyahia et al., 2004), the chemical composition of the leaves of *Eucalyptus cladocalyx* has been examined. Previous studies revealed the presence of various monoterpenes (He et al., 2000; Foudil-Cherif et al., 2000) and cyanogenic glycosides (Gleadow and Woodrow, 2000; Finnemore et al., 1936). This paper describes the isolation of a pentacyclic *nor*-triterpene, 17β-formyloxy-28-*nor*-urs-12-en-3β-ol, named cladocalol (**1**), together with five known compounds. The known compounds were identified by comparison of their spectral properties with those reported in the literature. We also investigated the biological effects of **1** and its

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0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.12.018

derivatives **2** and **3** on the growth of the human myeloid leukemia cell line HL-60. We found that these compounds exhibit cytotoxic activities as revealed by the MTT assay.

2. Results and discussion

The $CHCl_3$ extract of the leaves of a collection of *E. cladocalyx* from Algeria yielded 17β-formyloxy-28-*nor*-urs-12-en-3β-ol, named cladocalol **1**, and five known compounds ursulolactone acetate (Katai et al., 1983), ursolic acid (Lee, 1998), β-sitosterol (Khare et al., 2002), acid 3β-acetate-12,20(29)-lupadien-28-oic (Ryu et al., 1994), and the flavonoid eucalyptine (Horn et al., 1964). Compound **1** gave IR absorption bands for a hydroxyl group at 3300 cm^{-1} and a carbonyl group at 1717 cm^{-1}. Its mass spectrum showed a peak at *m/z* 411 indicating the loss of a formyl group from the molecular ion *m/z* 456. The molecular formula could be surmised as $C_{30}H_{48}O_3$. The ^{13}C NMR (Table 1) and DEPT spectra exhibited 30 resonances, including 6× C, 8× CH, 9× CH_2 and 7× CH_3 including two *sec*-methyls (δ_C 16.96, H_3-26; δ_C 17.25, H_3-29) and five *tert*-methyls (δ_C 15.43, H_3-26; δ_C 15.60, H_3-24; δ_C 20.49, H_3-25; δ_C 23.34, H_3-27; δ_C 28.12, H_3-23). The presence of a singlet at δ_H 8.00 in the ^1H NMR spectrum and doublet at δ_C 160.40 in the ^{13}C NMR spectrum clearly indicated the presence of a formyloxy group and this was confirmed by the HSQC correlation. The olefinic resonances at δ_C 137.72 and 126.04, corresponding to quaternary and methine carbons, suggested the

Table 1
^1H, ^{13}C NMR data of compounds **1**–**3**[a] [$CDCl_3$, *J* values (Hz) are given in parentheses]

No.	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	38.57	1.68 *m*	38.77	1.66 *m*	38.25	1.66 *m*
2	27.16	1.76 *m*	27.24	1.65 *m*	23.52	1.65 *m*
3	79.00	3.22 *dd*; (4.94, 10.97)	79.00	3.22 *dd*; (4.57, 10.74)	80.90	4.49 br *t*; (8.51)
4	38.82		38.67		37.67	
5	55.25	0.75 *m*	55.24	0.74 br *d*; (11.70)	55.33	0.82 *m*
6	18.26	1.60 *m*	18.32	1.55 *m*	18.15	1.50 *m* 1.38 *m*
7	33.17	1.50 *m*	33.01	1.51 *m*	33.10	1.52 *m*
		1.38 *m*		1.40 *m*		1.40 *m*
8	39.76		39.84		39.77	
9	47.60	1.52 *m*	47.62	1.54 *m*	47.53	1.55 *m*
10	36.98		36.99		36.90	
11	23.34	1.90 *m*	23.58	1.94 *dd*; (3.82, 11.11)	23.33	1.90 *m*
		1.82 *m*				
12	126.04	5.20 *t*; (3.61)	127.80	5.29 *t*; (3.65)	126.00	5.20 br *s*
13	137.72		138.00		137.80	
14	41.67		41.88		41.66	
15	26.28	1.80 *m*	26.00	2.04 *m*	26.27	1.82 *m*
		1.10 *m*		1.04 *m*		1.10 *m*
16	25.36	2.10 *m*	28.44	2.04 *m*	25.37	2.10 *m*
				2.02 *m*		
17	87.50		72.10		87.50	
18	56.78	2.08 *m*	60.60	1.58 *m*	56.79	2.10 *m*
19	38.72	1.30 *m*	39.28	1.66 *dd*; (3.65, 2.74)	38.83	1.35 *m*
20	41.33	1.47 *m*	41.56	1.28 *m*	41.33	1.42 *m*
21	32.11	1.65 *m*	32.33	1.60 *m*	32.11	1.62 *m*
		1.23 *m*		1.17 *m*		1.20 *m*
22	36.21	2.34 *dt* (3.20, 6.47)	40.41	1.72 *dt* (3.91, 6.32)	36.23	2.34 *dt* (3.22, 6.49)
		1.94 *m*		1.53 *m*		1.90 *m*
23	28.12	1.00 *s*	28.16	1.00 *s*	29.67	0.86[b]
24	15.60	0.76 *s*	15.62	0.79 *s*	16.72	0.86[b]
25	20.49	0.96 *s*	20.70	0.93 *s*	20.50	0.93 *s*
26	15.43	0.96 *s*	17.13	0.99 *s*	15.49	0.96 *s*
27	23.34	1.08 *s*	23.05	1.08 *s*	23.33	1.07 *s*
29	17.25	0.85 *d*; (6.48)	17.29	0.82 *d*; (6.44)	17.30	0.86[b]
30	16.96	0.95 *d*; (6.48)	15.49	0.94 *d*; (5.58)	16.96	0.86[b]
1′	160.49	8.00 *s*	–	–	160.50	8.00 *s*
C=O	–	–	–	–	171.00	
Oac	–	–	–	–	21.28	2.01 *s*

[a] Assignments are based on ^1H, ^{13}C, DEPT, ^1H–^1H COSY, HSQC, HMBC experiments.
[b] Overlapped signals.

presence of unsaturation such as C-12 of the ursane skeleton (Su et al., 2003). The absence of a peak for a CH_3-28 and the presence of a singlet at δ_C 87.50 suggested the assignment of –OCHO at the C-17 position. The position of the group, assigned to C-17 (δ_C 87.50), was established through long-range HMBC correlations with H-18 (δ_H 2.08), H-19 (δ_H 1.30) and H_2-22 (δ_H 1.94 and 2.34). A carbinolic methine proton at δ_H 3.22 correlated with δ_C 79.00 in a HSQC experiment indicated the presence of a hydroxyl group. It was placed at position 3 on biogenetic grounds. Its β configuration was assigned on the basis of chemical shifts and the coupling constant of H-3 (Lima et al., 2003).

When **1** was hydrolysed with $NaHCO_3$ a product (**2**) was obtained in which the formate signals were lost. Compound **1** underwent facile acetylation with Ac_2O/pyridine to give a crystalline monoacetate (**3**). The molecular structure and relative configuration of **3** were established by X-ray diffraction analysis. The structure was solved by direct methods using SIR97 (Altomare et al., 1999). Refinement was performed with SHELXL-93 (Sheldrick, 1993) using full-matrix least squares. The C31, C32, O1 and O2 belonging to the acetyl moiety and the C3 carbon ring through which this group is attached to the pentacyclic skeleton are disordered between two positions (65/35 population ratio) and were refined isotropically. All the remaining non-hydrogen atoms were anisotropically refined. The hydrogen atoms were placed at idealized positions and refined using a riding model. The final refinement converged at $R_1 = 8.93\%$ and $wR_2 = 17.93\%$, with a goodness of fit of 1.07 for 2160 reflections with $F_o > 4\sigma(F_o)$ and 325 parameters. The largest peak and hole on the final difference map were 0.34 and -0.20 e/Å^3, respectively. Fig. 1 shows a computer-generated perspective (Spek, 1992) view (at 50% of probability) of the final X-ray model of **3**. Based on **3**, the structure of cladocalol **1** is 17β-formyloxy-28-*nor*-urs-12-ene-3β-ol.

Ursane triterpenoids without the CH_3-28 carbon, such as **1**, are rare, and few compounds of this type have been synthesized (Tkachev and Denisov, 1994; Tatarov and Tkachev, 2001). To the best of our knowledge, compound **1** is the first example of a 28-*nor*-urs-12-ene isolated from natural source.

Triterpenoids are known to be anti-inflammatory and anticarcinogenic (Nishino et al., 1988; Huang et al., 1994). The effect of cladocalol and its derivatives on growth inhibition of the human tumor cell line HL-60 was also investigated. Cells were cultured for 72 h in the presence of increasing concentrations of the above compounds and metabolic activity was determined by the MTT procedure as described in the experimental section. Compounds **1** and **2** displayed similar cytotoxic potency, with IC_{50} of 42 ± 4 and 51 ± 1 μM, respectively (Table 2). These results indicate that the formyloxy group is not important for the cytotoxicity of cladocalol. However, acetylation of cladocalol to give the β-monoacetate derivative (compound **3**) increased twofold the IC_{50}, which suggests that the β-hydroxyl group seems to play an important role in its cytotoxic activity. Another explanation is also possible since the introduction

Table 2
Effects of cladocalol and its derivatives on the growth of HL-60 cells[a]

Compound	IC_{50} (μM)
Cladocalol (**1**)	42 ± 4
2	51 ± 1
3	83 ± 24

[a] The data shown represent the mean ± SEM of two (**3**) or three (**1**) and (**2**) experiments with three determinations in each. The IC_{50} values were calculated using the methodology described in Section 3 from cells treated for 72 h.

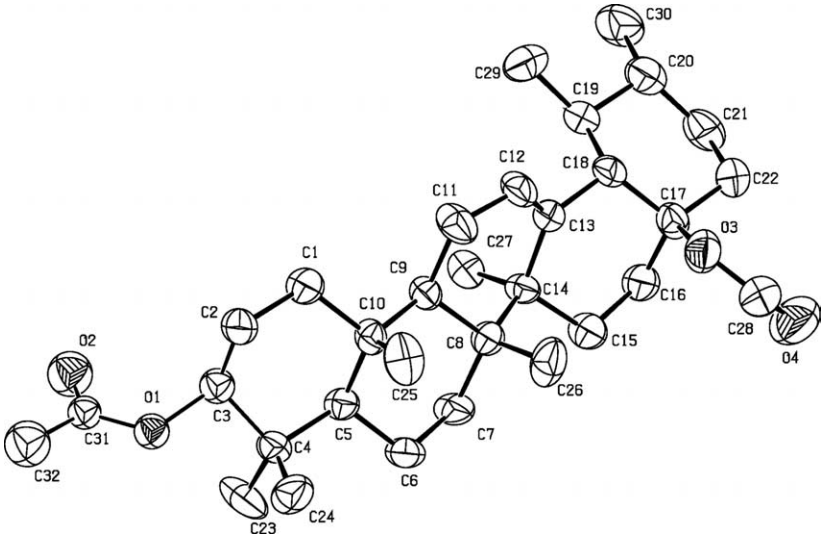


Fig. 1. ORTEP representation of 3 as determined by single-crystal X-ray analysis.

of a more bulky group (i.e., an acetyl group) could increase the steric hindrance, therefore decreasing the binding to the target molecule/s.

3. Experimental

3.1. General

Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Optical rotations were recorded in a Perkin–Elmer model 343 polarimeter. IR spectra were recorded using a Bruker model IFS-55 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker model AMX-400 and AMX-500 spectrometer with standard pulse sequences operating at 400 and 500 MHz in ^{1}H and 100 and 125 MHz in ^{13}C NMR. $CDCl_3$ was used as solvent. EIMS and HRE-IMS were taken on a Micromass model Autospec (70 eV) spectrometer. Column chromatography (CC) was carried out on silica gel 60 (Merck 230–400 mesh), and prep. TLC on silica gel 60 $PF_{254+366}$ plates (20×20 cm, 1 mm thickness).

3.2. Plant material

Leaves of *Eucalyptus cladocalyx* were collected in Taref Natural Park, Eastern Algeria, in June 1998 and identified by the Botany Department of INRF (El Kala). A voucher (158) specimen has been deposited in the herbarium of the Department of Forestry, University of Constantine.

3.3. Extraction and isolation

Dried leaves (1.5 kg) were repeatedly extracted with $CHCl_3$ at room temperature. The extracts were concentrated to dryness under reduced pressure. The residue (50 g) was chromatographed on Silica gel using hexane containing increasing amount of ethyl acetate to furnishing total 58 fractions that were obtained by combining the eluates on the basis of TLC. Fraction 11 (527 mg, *n*-hexane–EtOAc: 92%) was rechromatographed by CC on silica gel using *n*-hexane–$CHCl_3$–EtOAc, (8:1:1) to afford ursulolactone acetate (45 mg). Fraction 13 (3 g, *n*-hexane–EtOAc: 90%) was subjected to silica gel CC, using *n*-hexane–$CHCl_3$–EtOAc (16:16:1) to give two subfractions A and B. Subfraction A (350 mg) was rechromatographed on silica gel by CC using *n*-hexane–CH_2Cl_2–EtOAc, (16:16:1) and showed several spots on TLC which were separated through prep. TLC on silica gel using (*n*-hexane–CH_2Cl_2–EtOAc, 16:16:1) to afford **1** as a major compound (20 mg). Subfraction B (80 mg) was rechromatographed on silica gel using *n*-hexane–CH_2Cl_2–EtOAc, (8:8:1). Various fractions were also obtained and combined on the basis of TLC, affording ursolic aldehyde (12 mg) and β-sitosterol (27 mg), after purification on prep. TLC (*n*-hexane–CH_2Cl_2–EtOAc, 8:8:1). Fraction 16 (150 mg, *n*-hexane–EtOAc: 87%) afforded eucalyptine (40 mg) as a yellow solid, and fraction 30 (54 mg, *n*-hexane–EtOAc: 85%) was acetylated and chromatographed by CC using *n*-hexane–CH_2Cl_2–EtOAc, (16:1:1), to afford acid 3β-acetate-12,20(29)-lupadien-28-oic acid (12 mg).

3.4. Cladocalol (*1*)

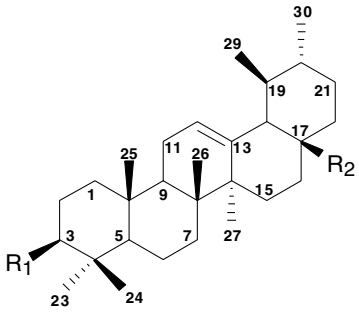
Obtained as an amorphous solid; $[\alpha]_D^{20}$ +58° ($CHCl_3$; *c* 0.2). IR ν_{max}^{KBr} cm^{-1}: 3300, 2926, 2871, 1717, 1508, 1386, 1195, 1042, 996, 732. EI-MS *m/z* (rel. int.): 411 (14), 410 (47), 392 (94), 377 (16), 349 (13), 255 (10), 241 (13), 215 (18), 202 (56), 189 (100), 173 (31), 145 (32), 133 (33), 119 (48) 105 (39). HR-EIMS *m/z* 411.3591 $[M–OCHO]^+$ (calcd. for $C_{29}H_{47}O_1$, 411.3626). ^{1}H and ^{13}C NMR see Table 1.

3.5. Hydrolysis of *1*

Cladocalol **1** (10 mg) was dissolved in 3 ml of MeOH, and was warmed with $NaHCO_3$ (10 mg) on a water bath (5 min). The mixture was chilled in ice-water and extracted thrice with $CHCl_3$. The solution was concentrated to dryness in vacuo. Prep. TLC with *n*-hexane–EtOAc (7:3) yielded 28-*nor*-urs-12-ene-3β, 17β-diol (**2**) (4 mg): amorphous solid; $[\alpha]_D^{20}$ +26° ($CHCl_3$; *c* 0.02). IR ν_{max}^{KBr} cm^{-1}: 3391, 2924, 2855, 1455, 1386, 1184, 1027, 994, 808, 732. EI-MS *m/z* (rel. int.): 428 (1), 410 (39), 392 (100), 377 (16), 349 (12), 241 (12), 215 (17), 202 (79), 189 (87), 173 (26), 161 (18), 145 (27), 132 (33), 119 (418) 104 (33); HR-EIMS *m/z* 428.3662 $[M]^+$ (calcd. for $C_{29}H_{48}O_2$, 428.3654).^{1}H and ^{13}C NMR see Table 1.

3.6. Acetylation of *1*

To a solution of 5 mg of cladocalol **1** in 1.5 ml pyridine, 2 ml acetic anhydride was added at 25 °C. After 10 h, the reaction was quenched with a dil. $NaHCO_3$ solution. Excess pyridine was eliminated by washing with aqueous $CuSO_4$. After extraction with $CHCl_3$ and purification on silica gel, 17β-formyloxy-3β-acetyloxy-28-*nor*-urs-12-ene **(3)** was obtained (5 mg): colourless needles (MeOH) mp 203–204 °C; $[\alpha]_D^{20}$ +59° ($CHCl_3$; *c* 0.05). IR ν_{max}^{KBr} cm^{-1}: 2926, 2873, 1719, 1456, 1370, 1246, 1193, 1026, 901, 732. EI-MS *m/z* (rel. int.): 452 (48), 392 (66), 377 (24), 349 (26), 255 (12), 241 (15), 239 (21), 215 (20), 202 (52), 189 (100), 175 (31), 173 (30), 159 (23), 145 (34), 133 (31), 119 (49) 105 (37). HR-EIMS *m/z* 452.3651 $[M–CO_2H_2]^+$ (calcd. for $C_{31}H_{48}O_2$, 452.3654). ^{1}H and ^{13}C NMR see Table 1.



1, R_1= OH; R_2= OCHO
2, R_1= OH; R_2= OH
3, R_1= OAc; R_2=OCHO

3.7. X-ray crystallography data of *3*

$C_{32}H_{50}O_4$, mol. wt. = 498.7, monoclinic, space group $P2_1$, $a = 8.674(2)$, $b = 8.162(2)$, $c = 20.744(4)$ Å, $\beta = 100.70(3)°$ $V = 1443.1(6)$ Å^3, $Z = 2$, Dc = 1.15 g cm^{-3}, $F(000) = 548$, μ = (Mo Kα) = 0.07 mm^{-1}. A single crystal of approximate dimensions 0.35×0.15×0.15 mm was used for all X-ray measurements. The intensity data of all unique reflections within the θ range 2.7–27.8° were collected at 273 K in an Enraf-Nonius Kappa CCD diffractometer, using graphite-monochromated Mo Kα ($\lambda = 0.71070$ Å) radiation. A total of 3476 unique reflections were recorded, of which 2160 with $F_o > 4\sigma(F_o)$ were taken into account for structure solution and refinements. Data reduction and cell parameters refinement were carried out with the programs COLLECT (Nonius Kappa, 1998) and DENZO (Otwinowski and Minor, 1997). Crystallographic data of **3**, including atomic coordinates, have been deposited with the Cambridge Crystallographic Data Centre (deposit number 253049). Copies of the data can be obtained free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: 44 1223 306033 or e-mail: deposit@ccdc.cam.ac.uk].

3.8. Cell culture

HL-60 cells were cultured in suspension in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were maintained at a density of $<1 \times 10^6$ cells/ml. Stock solutions of 10 mM compounds were made in DMSO, and aliquots were frozen at −20 °C. Further dilutions were made in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 0.5% (v/v), a concentration that was nontoxic to the cells. Cell viability was determined using the trypan blue exclusion test. To ensure an exponential growth, cells were resuspended in fresh medium 24 h before each treatment.

3.9. Cytotoxicity assay

Cytotoxic studies were performed using the MTT assay. Cells (1×10^4/well) were continuously exposed to different concentrations of the compounds in 96-well plates for 72 h at 37 °C. Controls were always treated with the same amount of DMSO as used in the corresponding experiments. Surviving cells were detected on the basis of their ability to metabolize 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan crystals. Optical density at 570 nm was used as a measure of cell viability. Cell survival was calculated as the fraction of cells alive relative to control for each point as cell survival (%) mean absorbance in treated wells/mean absorbance in control wells × 100. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment using the curve-fitting routine of the computer software Prism 2.0 (GraphPad) and the equation established by De Lean et al. (1978).

Acknowledgements

The authors thank the Direccion General de Universidades e Investigación (Canary Islands Goverment) for a Research Grant (ULPGC-INFR 2003/10 to F.E.) and ICIC (G-04-09 to J.B.)

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