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New polyoxygenated steroids from the Antarctic octocoral *Dasystenella acanthina*

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Abstract

The chemical study of the Antarctic octocoral *Dasystenella acanthina* has led to the isolation of the new polyoxygenated steroids (24*R*,22*E*)-24-hydroxycholest-4,22-dien-3-one (**1**), 23-acetoxy-24,25-epoxycholest-4-en-3-one (**2**), 12β-acetoxycholest-4-en-3,24-dione (**3**), 12β-acetoxy-24,25-epoxycholest-4-en-3-one (**4**), (22*E*)-25-hydroxy-24-norcholest-4,22-dien-3-one (**5**), 3α-acetoxy-25-hydroxycholest-4-en-6-one (**6**), and 3α,11α-diacetoxy-25-hydroxycholest-4-en-6-one (**7**), whose structures have been established by spectroscopic analysis. The absolute stereochemistry at C-24 in compound **1** has been determined through the ^{1}H NMR study of the corresponding (*R*)- and (*S*)-MPA esters. All the new compounds showed significant activities as growth inhibitors of several human tumor cell lines. In addition, cytostatic and cytotoxic effects were also observed on selected tumor cell lines.

Keywords: Steroids; Octocorals; *Dasystenella acanthina*; Structure elucidation; Cytotoxicity

1. Introduction

Along the last decades marine organisms have shown to be a major source of new steroidal metabolites displaying unprecedented structures. In particular, the chemical studies of marine octocorals have led to the discovery of a vast array of new steroid derivatives possessing multiple oxygenated functions at the carbocyclic nucleus and/or the side chain. Most of these studies have been focused on species from tropical and temperate waters where these organisms are widely distributed [1,2].

In contrast with earlier proposals, in recent years it has been claimed that the extreme and often unique conditions of the Antarctic marine environment, as well as the uncommon trophic interactions among its communities, could have led to organisms equipped with new bioactive metabolites [3,4]. However, also likely due to the difficult access to Antarctic organisms, the reported accounts on new natural products from Antarctic octocorals have been limited to a series of sesquiterpenes isolated from the species *Alcyonium paessleri* [5,6] *Ainigmaptilon antarcticus* [7], and *Dasystenella acanthina* [8]. To the best of our knowledge, the only report on steroids from Antarctic octocorals describes the isolation and ecological roles of cholesterol and its 24-methylen-, 22-dehydro- and 22-dehydro-7β-hydroxy derivatives obtained from *A. paessleri* [9].

As a part of our project directed towards the search for bioactive metabolites from marine octocorals, we have examined specimens of the gorgonian octocoral *D. acanthina* (Wright and Studer, 1889) collected in the Eastern Weddell Sea (Antarctica). This study has led to the isolation of the new polyoxygenated steroids **1**–**7**, together with the known compounds **8** [10], which has been encountered for the first time from natural sources, and *trans*-β-farnesene (8). From a structural point of view, the presence of an oxygenated function at C-12 in compounds **3** and **4** is a feature rarely found in natural steroids [11–18]. On the other hand, the Δ^4-3-acetoxy-6-keto moiety present in compounds **6** and **7** has only been described in synthetic derivatives [19–21].

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2. Experimental

2.1. General methods

Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were obtained on a Philips PU 8710 spectrophotometer and IR spectra were recorded on a Genesis Series FT IR Mattson spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 400 or on a Varian INOVA 600 spectrometer using $CDCl_3$ as solvent. Proton chemical shifts were referenced to the residual $CHCl_3$ signal at δ 7.26, and ^{13}C NMR spectra were referenced to the central peak of $CDCl_3$ at δ 77.0. NOESY experiments, ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) were performed using standard VARIAN pulse sequences. Low resolution mass spectra were recorded on a Finnigan Voyager GC8000^{top} spectrometer. High resolution mass spectra (HRMS) were obtained on a VG Autospec spectrometer. Column chromatography was carried out on Merck Silica gel 60 (70–230 mesh). HPLC separations were performed on a LaChrom-Hitachi apparatus equipped with LiChrospher Si-60 (Merck) columns in normal phase mode and LiChrosorb RP-18 (Merck) columns in reversed phase mode using a differential refractometer RI-71. All solvents were spectral grade or were distilled prior to use.

2.2. Biological material

Specimens of *D. acanthina* (Wright and Studer, 1889) were collected on the *R/V Polarstern* cruises ANT XVII/3 (EASIZ-III, Ecology of Antarctic Sea Ice Zone, March 17–May 11, 2000) sponsored by the Alfred Wegener Institut für Polar und Meeresforschung (Bremerhaven) under the auspices of the Scientific Committee for Antarctic Research (SCAR). All the material examined of *D. acanthina* was collected using a bottom trawl off Kapp Norvegia (Eastern Weddell Sea, Antarctica) in April 2000. The colonies were sorted on board and washed in running sea water to avoid external contamination from other organisms collected in the same haul, and immediately frozen at −27 °C. Taxonomy: Phylum Cnidaria, Class Anthozoa, Subclass Octocorallia, Order Alcyonacea, Family Primnoidae.

2.3. Extraction and isolation

Frozen specimens of *D. acanthina* (112.0 g) were chopped and extracted with 5 L of acetone/methanol (1:1) at room temperature. After filtration of the solvent, the solution was evaporated under reduced pressure to obtain an aqueous residue that was partitioned between H_2O and Et_2O. The organic layer was taken to dryness to give an orange oil (1.83 g), that was chromatographed on a SiO_2 column using solvents of increasing polarities from hexane to Et_2O, then $CHCl_3$/MeOH (8:2), and finally MeOH. The fraction eluted with hexane/Et_2O (6:4) was subjected to normal phase HPLC with hexane/EtOAc (75:25) yielding (24*R*,22*E*)-24-hydroxycholest-4,22-dien-3-one (**1**, 4.5 mg, 0.004% dry wt.) and 23-acetoxy-24,25-epoxycholest-4-en-3-one (**2**, 1.5 mg, 0.001% dry wt.). The fraction of the general chromatography eluted with hexane/Et_2O (1:1) was subjected to HPLC with hexane/EtOAc (75:25) yielding 12β-acetoxycholest-4-en-3,24-dione (**3**, 34.3 mg, 0.030 dry wt.), 3α-acetoxy-25-hydroxycholest-4-en-6-one (**6**, 1.0 mg, 0.0008% dry wt.) and (22*E*)-25-hydroxy-24-nor-cholest-4, 22-dien-3-one (**5**, 7.5 mg, 0.007% dry wt.). The fraction eluted with hexane/Et_2O (3:7) was subjected to HPLC using hexane/EtOAc (7:3) and the more polar fraction obtained was purified by reversed-phase HPLC using MeOH/H_2O (85:15) to give 12β-acetoxy-24,25-epoxycholest-4-en-3-one (**4**, 1.0 mg, 0.0008% dry wt.) and 25-hydroxycholest-4-en-3-one (**8**, 1.5 mg, 0.001% dry wt.). Finally, the fraction eluted with hexane/Et_2O (1:9) was purified by HPLC with hexane/EtOAc (65:35) yielding 3α,11α-diacetoxy-25-hydroxycholest-4-en-6-one (**7**, 2.67 mg, 0.002% dry wt.). Final purification of **7** was afforded by RP-18 cartridge eluted with MeOH.

2.3.1. (24R, 22E)-24-Hydroxycholest-4,22-dien-3-one (*1*)

White amorphous powder, $[\alpha]_D^{25} = +47.6$ ($c = 0.2$, $CHCl_3$), UV (MeOH) 240 ($\varepsilon = 13{,}200$). IR (film): 3468, 2944, 2868, 1678, 1240 cm^{-1}. EIMS (70 eV): *m/z* (%): 398 (7.8), 355 (100.0), 271 (33.9), 147 (43.3). HRCIMS *m/z* = 399.3249, calculated for $C_{27}H_{43}O_2$ $(M + H)^+$ 399.3263. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.3.2. 23-Acetoxy-24,25-epoxycholest-4-en-3-one (*2*)

White amorphous powder, $[\alpha]_D^{25} = +51.4$ ($c = 0.12$, $CHCl_3$), UV (MeOH) 239 ($\varepsilon = 10{,}500$). IR (film): 2939, 2859, 1738, 1673, 1237 cm^{-1}. EIMS (70 eV): *m/z* (%): 457 (16.2), 398 (10.0), 271 (24.5), 147 (58.2), 124 (82.9). HRCIMS *m/z* = 457.3317, calculated for $C_{29}H_{45}O_4$ $(M + H)^+$ 457.3318. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.3.3. 12β-Acetoxycholest-4-en-3,24-dione (*3*)

White amorphous powder, $[\alpha]_D^{25} = +68.7$ ($c = 0.1$, $CHCl_3$), UV (MeOH) 239 ($\varepsilon = 9380$). IR (film): 2956, 2874, 1737, 1676, 1240 cm^{-1}. EIMS (70 eV): *m/z* (%): 457 (10.5), 396 (23.0), 269 (67.0), 127 (90.3), 71 (100). HRCIMS *m/z* = 457.3329, calculated for $C_{29}H_{45}O_4$ $(M + H)^+$ 457.3318. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.3.4. 12β-Acetoxy-24,25-epoxycholest-4-en-3-one (*4*)

White amorphous powder, $[\alpha]_D^{25} = +71.8$ ($c = 0.03$, $CHCl_3$), UV (MeOH) 239 ($\varepsilon = 16{,}830$). IR (film): 2940,

Table 1
^{1}H NMR data for compounds **1**–**7**[a]

Carbon atom	**1**[b]	**2**[b]	**3**[b]	**4**[c]	**5**[b]	**6**[c]	**7**[b]
1	1.68 (m)α, 2.01 (m)β	1.68 (m)α, 2.00 (m)β	1.70 (m)α, 1.97 (ddd, 13.3, 5.1, 3.3)β	1.70 (m)α, 1.95 (ddd, 13.4, 5.0, 3.2)β	1.69 (m)α, 2.01 (m)β	1.57 (m)α, 1.66 (ddd, 13.4, 3.9, 3.3)β	1.48 (m)β, 1.85 (m)α
2	2.36 (m), 2.42 (m)	2.36 (m), 2.40 (m)	2.33 (m), 2.41 (m)	2.32 (m), 2.38 (m)	2.34 (m), 2.42 (m)	1.84 (m)	1.82 (m)
3	–	–	–	–	–	5.27 (m)	5.22 (bt, 4.3)
4	5.72 (bs)	5.72 (bs)	5.72 (bs)	5.73 (bs)	5.72 (bs)	6.22 (d, 4.6)	6.32 (dd, 4.9, 1.3)
5	–	–	–	–	–	–	–
6	2.26 (ddd, 14.6, 4.1, 2.5)α, 2.33 (m)β	2.26 (ddd, 14.5, 3.8, 2.4)α, 2.38 (m)β	2.27 (ddd, 14.8, 4.1, 2.2)α, 2.37 (m)β	2.28 (ddd, 15.0, 4.3, 2.4)α, 2.36 (m)β	2.25 (ddd, 14.2, 4.1, 2.3)α, 2.38 (ddd, 14.2, 14.2, 5.0)β	–	–
7	1.00 (m)α, 1.83 (m)β	1.02 (m)α, 1.83 (m)β	0.98 (m)α, 1.83 (m)β	1.00 (m)α, 1.84 (m)β	1.02 (m)α, 1.83 (m)β	1.96 (dd,16.0, 12.4)α, 2.57 (dd, 16.0, 4.4)β	2.06 (m)α, 2.59 (dd, 15.7, 3.9)β
8	1.52 (m)	1.48 (m)	1.48 (m)	1.48 (m)	1.52 (m)	1.84 (m)	1.91 (m)
9	0.92 (m)	0.92 (m)	1.08 (m)	1.08 (m)	0.92 (m)	1.30 (m)	1.73 (t, 10.5)
10	–	–	–	–	–	–	–
11	1.42 (m)β, 1.50 (m)α	1.38 (m)β, 1.50 (m)α	1.42 (m)β, 1.75 (m)α	1.42 (m)β, 1.75 (m)α	1.44 (m)β, 1.51 (m)α	1.46 (m)β, 1.63 (m)α	5.28 (ddd, 10.5, 10.5, 5.3)
12	1.18 (m)α, 2.01 (m)β	1.18 (m)α, 2.00 (m)β	4.64 (dd, 11.1, 4.9)	4.66 (dd, 11.0, 4.9)	1.18 (m)α, 2.02 (m)β	1.22 (m)α, 2.07 (ddd, 12.9, 3.6, 3.3)β	1.25 (m)α, 2.37 (dd, 12.2, 5.3)β
13	–	–	–	–	–	–	–
14	1.02 (m)	1.02 (m)	1.05 (m)	1.05 (m)	1.02 (m)	1.14 (m)	1.25 (m)
15	1.08 (m), 1.58 (m)	1.14 (m),1.58 (m)	1.24 (m), 1.67 (m)	1.25 (m), 1.68 (m)	1.10 (m), 1.58 (m)	1.10 (m), 1.59 (m)	1.60 (m), 1.10 (td, 12.0, 6.2)
16	1.26 (m), 1.68 (m)	1.26 (m), 1.86 (m)	1.52 (m), 1.75 (m)	1.51 (m), 1.78 (m)	1.26 (m), 1.66 (m)	1.26 (m), 1.86 (m)	1.30 (m), 1.88 (m)
17	1.12 (m)	1.10 (m)	1.42 (m)	1.42 (m)	1.16 (m)	1.14 (m)	1.18 (m)
18	0.73 (s)	0.70 (s)	0.85 (s)	0.84 (s)	0.72 (s)	0.75 (s)	0.78 (s)
19	1.18 (s)	1.18 (s)	1.17 (s)	1.17 (s)	1.18 (s)	0.95 (s)	1.07 (s)
20	2.11 (m)	1.39 (m)	1.55 (m)	1.62 (m)	2.05 (m)	1.42 (m)	1.38 (m)
21	1.03 (d, 6.7)	0.96 (d, 6.4)	0.85 (d, 6.8)	0.89 (d, 6.7)	1.02 (d, 6.8)	0.94 (d, 6.6)	0.92 (d, 6.8)
22	5.49 (ddd, 15.3, 8.3, 0.7)	1.06 (m), 1.82 (m)	1.20 (m), 1.80 (m)	1.06 (m), 1.68 (m)	5.43 (dd, 15.7, 8.4)	1.06 (m), 1.37 (m)	1.03 (m), 1.34 (m)
23	5.38 (dd, 15.3, 6.9)	4.93 (ddd, 10.8, 8.4, 2.6)	2.37 (m), 2.47 (ddd, 16.9, 9.4, 5.3)	1.40 (m), 1.58 (m)	5.52 (d, 15.7)	1.22 (m), 1.37 (m)	1.38 (m)
24	3.77 (ddd, 6.7, 6.1, 0.7)	2.78 (d, 8.4)	–	2.68 (dd, 6.7, 5.8)		1.46 (m), 1.37 (m)	1.34 (m), 1.43 (m)
25	1.68 (m)	–	2.59 (h, 6.9)	–	–	–	–
26	0.91 (d, 6.8)	1.32 (s)	1.08 (d, 7.2)	1.30 (s)	1.29 (s)	1.22 (s)	1.21 (s)
27	0.87 (d, 6.8)	1.35 (s)	1.08 (d, 7.2)	1.25 (s)	1.29 (s)	1.22 (s)	1.21 (s)
OAc		2.09 (s)	2.02 (s)	2.02 (s)		2.05 (s)	2.05 (s)
OAc							2.06 (s)

[a] Spectra recorded in $CDCl_3$. *J* in Hz. Assignments aided by COSY, HSQC, HMBC, and NOESY experiments.
[b] Spectra recorded at 400 MHz.
[c] Spectra recorded at 600 MHz.

Table 2
^{13}C NMR data for compounds **1**–**7**[a]

Carbon atom	**1**[b]	**2**[b]	**3**[b]	**4**[c]	**5**[b]	**6**[c]	**7**[b]
1	35.6	35.6	35.6	35.6	35.6	30.8	31.2
2	33.9	33.9	33.8	33.8	33.9	24.3	24.4
3	199.6	199.8	199.2	199.2	199.6	65.3	64.9
4	123.8	123.8	124.2	124.2	123.7	126.0	128.0
5	171.5	171.4	170.1	170.1	171.5	150.9	149.7
6	32.9	32.9	32.7	32.7	32.9	203.4	200.1
7	32.0	32.0	31.2	31.2	32.0	46.4	46.0
8	35.7	35.7	34.3	34.3	35.7	34.0	32.9
9	53.8	53.7	52.1	52.1	53.8	50.8	53.0
10	38.6	38.4	38.4	38.4	38.6	38.7	39.8
11	21.0	21.0	27.2	27.2	21.0	21.4	70.6
12	39.5	39.5	80.7	80.7	39.5	39.4	46.2
13	42.4	42.5	46.2	46.2	42.3	42.6	42.6
14	55.8	56.2	53.8	53.9	55.9	56.6	55.3
15	24.2	24.4	23.6	23.7	24.0	23.9	23.9
16	28.5	28.4	24.4	24.4	28.5	28.0	28.1
17	55.7	55.9	56.5	56.6	55.7	56.0	55.8
18	12.2	11.9	8.8	8.9	12.1	11.9	12.6
19	17.4	17.4	17.1	17.2	17.3	18.6	19.3
20	39.7	32.2	32.7	33.3	39.4	35.7	35.5
21	20.5	18.8	20.6	20.9	20.4	18.3	18.6
22	138.8	38.6	28.4	31.4	133.2	36.3	36.2
23	128.7	71.2	38.2	27.1	135.5	20.7	20.7
24	78.1	65.2	215.0	64.7		44.3	44.3
25	34.0	58.4	40.8	58.1	70.6	71.1	71.0
26	18.2d	24.7	18.2d	24.9	29.9d	29.4d	29.4d
27	18.1d	19.3	18.3d	18.7	29.8d	29.2d	29.2d
OCO*CH*$_3$		21.1	21.5	21.6		21.1	21.9e
O*CO*CH$_3$		170.5	170.5	170.5		170.3	170.2
OCO*CH*$_3$							21.2e
O*CO*CH$_3$							170.2

Values with the letters (d and e) in the same column are interchangeable.
[a] Spectra recorded in $CDCl_3$. Assignments aided by HSQC and HMBC experiments.
[b] Spectra recorded at 100 MHz.
[c] Spectra recorded at 150 MHz.

2866, 1752, 1685, 1240 cm^{-1}. EIMS (70 eV): *m/z* (%): 457 (15.3), 398 (25.0), 378 (29.8), 338 (30.3), 269 (59.0), 147 (43.8). HRCIMS *m/z* = 457.3324, calculated for $C_{29}H_{45}O_4$ $(M + H)^+$ 457.3318. ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.3.5. (22E)-25-Hydroxy-24-norcholest-4,22-dien-3-one (5)

White amorphous powder, $[\alpha]_D{}^{25} = +45.9$ (c = 0.07, $CHCl_3$), UV (MeOH) 240 (ε = 14,900). IR (film): 3445, 2943, 2867, 1673, 1230 cm^{-1}. EIMS (70 eV): *m/z* (%): 385 (31.4), 369 (35.7), 315 (100.0), 271 (56.6), 147 (75.3). HRCIMS *m/z* = 385.3117, calculated for $C_{26}H_{41}O_2$ $(M + H)^+$ 385.3107 ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.3.6. 3α-Acetoxy-25-hydroxycholest-4-en-6-one (6)

White amorphous powder, $[\alpha]_D{}^{25} = +76.5$ (c = 0.06, $CHCl_3$), UV (MeOH) 232 (ε = 6740). IR (film): 3480, 2939, 2867, 1740, 1696, 1237 cm^{-1}. EIMS (70 eV): *m/z* (%): 459 (6.4), 417 (100), 399 (9.2), 384 (10.5), 121 (59.8). HRCIMS *m/z* = 458.3395, calculated for $C_{29}H_{46}O_4$ $(M)^+$ 458.3396. ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.3.7. 3α,11α-Diacetoxy-25-hydroxycholest-4-en-6-one (7)

White amorphous powder, $[\alpha]_D{}^{25} = +62.4$ (c = 0.14, $CHCl_3$), UV (MeOH) 233 (ε = 7170). IR (film): 3466, 2950, 2867, 1737, 1676, 1244 cm^{-1}. EIMS (70 eV): *m/z* (%): 517 (6.1), 475 (100), 399 (13.7), 121 (64.7). HRCIMS *m/z* = 516.3445, calculated for $C_{31}H_{48}O_6$ $(M)^+$ 516.3451. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.4. Absolute configuration

2.4.1. Synthesis of the (R)-MPA ester of compound 1

Compound **1** (1.4 mg, 3.5×10^{-3} mmol) was treated with CH_2Cl_2 solutions of *N*,*N*′-dicyclohexylcarbodiimide (7.5 mg, 0.036 mmol in 0.5 mL), *N*,*N*-dimethylaminopyridine (1 mg, 8.2×10^{-3} mmol in 0.5 mL) and (*R*)-α-methoxyphen-

ylacetic acid (2 mg, 0.012 mmol in 0.5 mL) and the mixture was stirred at room temperature for 3 h. Evaporation of the solvent under reduced pressure yielded a residue that was purified by HPLC (Hexane/EtOAc 85:15) to obtain 0.4 mg (7.3×10^{-4} mmol) of the (*R*)-MPA ester **1a**: ^{1}H NMR (600 MHz, $CDCl_3$): (selected data, assignments aided by a COSY experiment) δ 5.73 (bs, 1H, H-4), 5.21 (dd, 1H, $J = 15.3$, 9.0, H-22), 5.07 (dd, 1H, $J = 15.3$, 7.5, H-23), 4.98 (dd, 1H, $J = 7.5$, 6.6, H-24), 1.89 (m, 1H, H-20), 1.82 (m, 1H, H-25), 1.17 (s, 3H, H-19), 0.86 (d, 3H, $J = 7.2$, H-26), 0.86 (d, 3H, $J = 7.2$, H-27), 0.85 (d, 3H, $J = 6.6$, H-21) and 0.63 (s, 3H, H-18).

*2.4.2. Synthesis of the (S)-MPA ester of compound **1***

Treatment of compound **1** (1.6 mg, 3.9×10^{-3} mmol) with CH_2Cl_2 solutions of *N*,*N*′-dicyclohexylcarbodiimide (7.5 mg, 0.036 mmol in 0.5 mL), *N*,*N*-dimethylaminopyridine (1 mg, 8.2×10^{-3} mmol in 0.5 mL) and (*S*)-α-methoxyphenylacetic acid (2 mg, 0.012 mmol in 0.5 mL) as described above (rt 3 h) yielded 2.1 mg (3.8×10^{-3} mmol) of the (*S*)-MPA ester **1b**: ^{1}H NMR (600 MHz, $CDCl_3$): (selected data, assignments aided by a COSY experiment) δ 5.72 (bs, 1H, H-4), 5.50 (dd, 1H, $J = 15.6$, 9.0, H-22), 5.25 (dd, 1H, $J = 15.6$, 7.8, H-23), 4.95 (dd, 1H, $J = 7.8$, 6.6, H-24), 2.03 (m, 1H, H-20), 1.72 (m, 1H, H-25), 1.18 (s, 3H, H-19), 0.98 (d, 3H, $J = 6.6$, H-21), 0.70 (d, 3H, $J = 7.2$, H-26), 0.70 (d, 3H, $J = 7.2$, H-27) and 0.69 (s, 3H, H-18).

2.5. Cytotoxic activity

All the compounds isolated from *D. acanthina* were tested against the following human tumor cell lines: DU-145 (prostate carcinoma), LN-caP (prostate carcinoma), IGROV (ovarian adenocarcinoma), SK-BR3 (breast adenocarcinoma), SK-MEL-28 (melanoma), A549 (lung adenocarcinoma), K-562 (chronic myelogenous leukemia), PANC1 (pancreas carcinoma), HT29 (colon adenocarcinoma), LOVO (colon adenocarcinoma), LOVO-DOX (colon adenocarcinoma resistant to doxorubicin), and HELA (cervix epithelial adenocarcinoma).

3. Results and discussion

Frozen specimens of *D. acanthina* were extracted with acetone–methanol (1:1). After evaporation of the solvent under reduced pressure, the aqueous residue was extracted with Et_2O and the organic extract subjected to column chromatography eluted with He/Et_2O mixtures, then $CHCl_3$/MeOH (8:2), and finally MeOH. The more polar fractions showed cytotoxic activity against the human tumor cell lines HT29, A549, and LOVO-DOX and were further separated on HPLC to yield compounds **1**–**8**.

Compound **1** was isolated as an amorphous solid whose molecular formula, $C_{27}H_{42}O_2$, was determined by HRMS. The ^{1}H NMR spectrum of compound **1** (Table 1) exhibited five signals at $\delta = 1.18$ (s, 3H), 1.03 (d, 3H, $J = 6.7$ Hz), 0.91 (d, 3H, $J = 6.8$ Hz), 0.87 (d, 3H, $J = 6.8$ Hz), and 0.73 (s, 3H) attributable to the methyl groups at C-19, C-21, C-26/C-27, and C-18, respectively, of a steroidal metabolite. These data, together with a general inspection of the ^{13}C NMR spectrum (Table 2) which accounted for the 27 carbons of the molecular formula, were consistent with a cholestane carbon framework [22]. Furthermore, the ^{13}C NMR signal at $\delta = 199.6$ (s) was attributable to the carbonyl group of an α,β-unsaturated ketone, while the signal at $\delta = 78.1$ (d) together with the IR absorption band at 3468 cm^{-1} indicated the presence of a secondary hydroxyl group in the molecule. The remaining functional groups of compound **1** were identified as two carbon-carbon double bonds, one of them conjugated with the ketone function, that gave rise to the ^{13}C NMR signals at $\delta = 171.5$ (s), 138.8 (d), 128.7 (d) and 123.8 (d). The location of all these functionalities on the cholestane framework was determined through the analysis of the COSY, HSQC and HMBC spectra.

Thus, the olefinic carbon signals at $\delta = 138.8$ and 128.7 were correlated in the HSQC spectrum with two proton signals at $\delta = 5.49$ (ddd, 1H, $J = 15.3$, 8.3, and 0.7 Hz) and 5.38 (dd, 1H, $J = 15.3$ and 6.9 Hz), respectively, assigned to two *trans*-coupled olefinic protons. On the cholestane skeleton, a *trans*-disubstituted double bond had to be located in the side chain. In fact, it was determined that the double bond was located at C-22,C-23 upon observation in the COSY spectrum of a cross peak between the olefinic proton signal at $\delta = 5.49$ and a methyne proton signal at $\delta = 2.11$ (m, 1H) which, in turn, was correlated with the Me-21 doublet at $\delta = 1.03$. On the other hand, the carbon signal at $\delta = 78.1$ was correlated in the HSQC spectrum with a proton signal at $\delta = 3.77$ (ddd, 1H, 6.7, 6.1, and 0.7 Hz) which in the COSY spectrum was coupled with the olefinic proton signal at $\delta = 5.38$ (H-23). These data defined the location of the secondary hydroxyl group at C-24.

The two remaining olefinic carbon signals above mentioned at $\delta = 171.5$ (s) and 128.7 (d) in the ^{13}C NMR spectrum, together with the proton signal at $\delta = 5.72$ (bs, 1H) in the ^{1}H NMR spectrum, were therefore due to a trisubstituted double bond conjugated with the ketone group. Taking into account the usual presence of an oxygenated function at C-3 in steroidal metabolites, it was proposed that the carbonyl group was located at C-3, and therefore the trisubstituted double bond at C-4,C-5. This proposal was confirmed from the correlations observed in the HMBC spectrum. In fact, the signal $\delta = 171.5$, assigned to the carbon in β-position with respect to the carbonyl, showed a cross peak with the Me-19 proton signal at $\delta = 1.18$ (s), while the carbonyl signal at $\delta = 199.6$ showed three-bonds correlations with the proton signals of a methylene at $\delta = 2.01$ (m, 1H, H-1) and 1.68 (m, 1H, H-1), which in turn were correlated with the Me-19 carbon signal at $\delta = 17.4$ (q).

All these data, together with a careful analysis of the COSY, HSQC and HMBC spectra that allowed us to fully assign the ^{1}H and ^{13}C NMR data of compound **1**, were in

agreement with the structure (22*E*)-24-hydroxycholest-4,22-dien-3-one.

The analysis of the NOESY spectrum confirmed that compound **1** possessed the usual stereochemistry of the hydrocarbon framework of the cholestanes [23]. With respect to the chiral center at C-24, its configuration was established through chemical derivatization of compound **1** with (*R*)- and (*S*)-α-methoxyphenylacetic acids (MPA) and subsequent analysis of the resulting diastereomeric esters **1a** and **1b** by ^{1}H NMR. Negative $\Delta\delta(\delta_R - \delta_S)$ values were found for H-20, H-21, H-22 and H-23, while positive $\Delta\delta$ values were obtained for H-25, Me-26 and Me-27 (Fig. 1). Following the MPA rules [24], these data indicated an *R* configuration for C-24. Therefore it was proposed the structure (24*R*, 22*E*)-24-hydroxycholest-4,22-dien-3-one for compound **1**.

Compound **2** was isolated as an amorphous solid of molecular formula $C_{29}H_{44}O_4$, as established by HRMS. The presence of an acetoxyl group was readily inferred from the NMR signals at $\delta_H = 2.09$ (s, 3H) and $\delta_C = 170.5$ (s) and 21.1 (q). The remaining 27 signals of the ^{13}C NMR spectrum were attributable to a cholestane skeleton. Furthermore, a comparison with the ^{13}C NMR data of compound **1** indicated that both compounds, **1** and **2**, shared an identical tetracyclic nucleus containing the same enone moiety. This structural assignment was further confirmed through the analysis of the COSY, HSQC and HMBC spectra, that also led to establish the structure of the side chain of compound **2** as follows.

The presence of three methyl groups at C-21, C-26 and C-27 was deduced from the ^{1}H NMR signals at $\delta = 0.96$ (d, 3H, $J = 6.4$ Hz), 1.32 (s, 3H), and 1.35 (s, 3H), respectively, with these two latter signals suggesting the presence of an oxygenated function at C-25. A signal at $\delta = 2.78$ (1H, d, $J = 8.4$ Hz) that was correlated in the HSQC spectrum with a carbon signal at $\delta = 65.2$ (d), was attributable to an oxymethyne proton of an epoxide ring. Furthermore, in the HMBC spectrum the proton singlets of the methyl groups at C-26 and C-27 showed cross peaks with the epoxide carbon signal at $\delta = 65.2$ consequently assigned to C-24, as well as with a singlet at $\delta = 58.4$, that had to be due to C-25. Taking into account the presence in the molecule of four oxygen atoms and seven unsaturations deduced from the molecular formula, all these data were in agreement with the presence of an epoxy function between C-24 and C-25. Finally, a signal in the ^{1}H NMR spectrum at $\delta = 4.93$ (ddd, 1H, $J = 10.8$, 8.4 and 2.6 Hz), that was correlated in the HSQC spectrum with the carbon signal at $\delta = 71.2$, was assigned to a proton geminal to the acetoxyl group. This

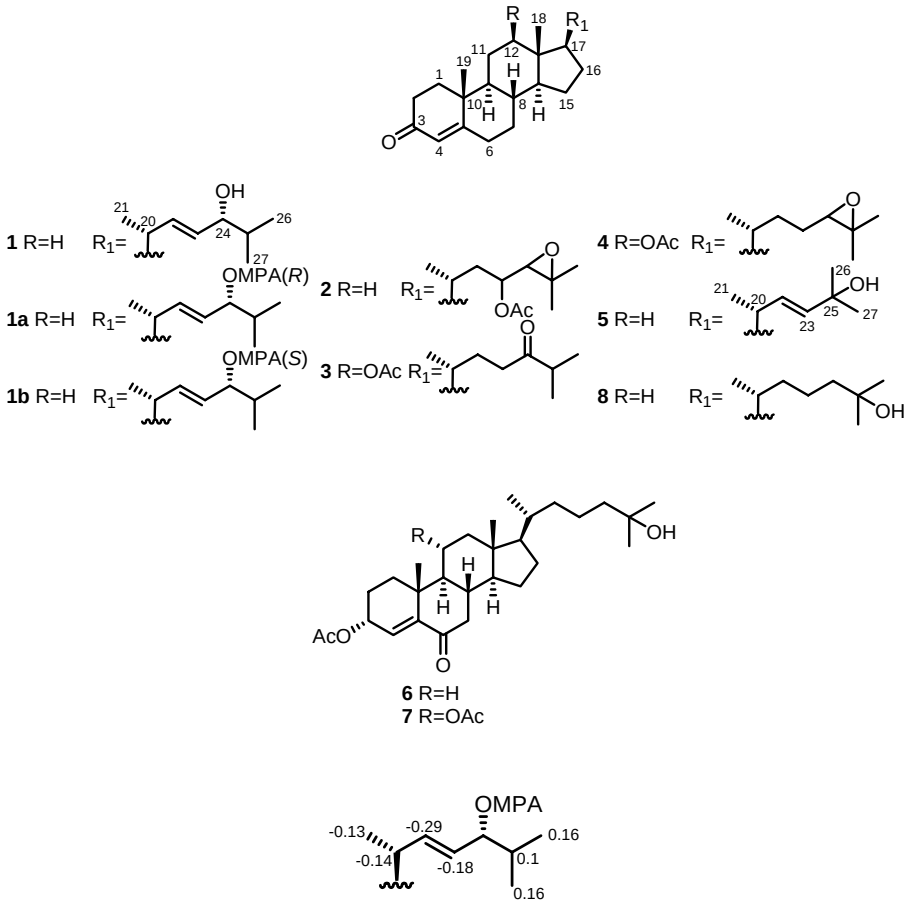

Fig. 1. Chemical shifts differences ($\Delta\delta = \delta_R - \delta_S$, in ppm) between the MPA esters **1a** and **1b**.

proton was readily identified as H-23 upon observation in the COSY spectrum of a cross peak with the signal at $\delta =$ 2.78 previously assigned to the proton H-24 on the oxirane ring.

Based on all this spectroscopic evidences it was proposed the structure 23-acetoxy-24,25-epoxycholest-4-en-3-one for compound **2**. Although the cholestane **2** contains two additional chiral centers at C-23 and C-24, their absolute configuration could not be investigated since the small amount of compound available prevented any chemical transformation.

The ^{13}C NMR spectrum of compound **3** displayed 29 signals, 2 of them [$\delta = 170.5$ (s) and 21.5 (q)] corresponding to an acetoxyl group and the remaining 27 attributable to a cholestane framework, whose presence was further ascertained by the ^{1}H NMR signals at $\delta = 1.17$ (s, 3H), 1.08 (d, 6H, $J = 7.2$) and 0.85 (s, 3H) due to the methyl groups at C-19, C-26/C-27, and C-18, respectively. The NMR spectra of compound **3** showed, as those of compounds **1** and **2**, the characteristic signals of the enone moiety of ring A: one olefinic proton signal at $\delta_H = 5.72$ (bs, 1H) and three carbon signals at $\delta_C = 199.2$ (CO), 124.2 (C-4) and 170.1 (C-5). A signal at $\delta = 215.0$ (s) in the ^{13}C NMR spectrum indicated the presence of an additional carbonyl group. This function was located at C-24 upon observation in the HMBC spectrum of a cross peak between this carbonyl signal and the proton signal at $\delta = 1.08$ due to the methyl groups at C-26 and C-27. On the other hand, it was deduced that the acetoxyl group was linked to a methyne group upon observation in the ^{1}H NMR spectrum of a signal at $\delta = 4.64$ (dd, 1H, $J = 11.1$, 4.9 Hz) that was correlated in the HSQC spectrum with a carbon signal at 80.7 (d). The analysis of the COSY, HSQC and HMBC spectra of compound **3** allowed to fully assign the NMR signals and to determine that the acetoxyl group was attached to C-12. Thus, in the HMBC spectrum the signal due to the proton geminal to the acetoxyl group ($\delta_H = 4.64$) was correlated with three carbon signals at $\delta = 56.5$ (d), 27.2 (t) and 8.8 ppm (q), corresponding to C-17, C-11 and C-18, respectively, while the signal due to the carbon bearing the acetoxyl group ($\delta_C = 80.7$) was correlated with the proton signal of Me-18 at $\delta = 0.85$ and with the signals assigned to H-11 protons at 1.75 (m, 1H) and 1.42 (m, 1H). Finally, the vicinal coupling constants of H-12 with the protons H-11 ($^{3}J_{11ax,12} = 11.1$ and $^{3}J_{11eq,12} = 4.9$ Hz) indicated an axial orientation of H-12 and therefore an equatorial or β orientation of the acetoxyl group. All these data led to propose the structure 12β-acetoxycholest-4-en-3,24-dione for compound **3**.

Compound **4** was isolated as an amorphous solid whose molecular formula $C_{29}H_{44}O_4$, determined by HRMS, indicated that it was an isomer of compounds **2** and **3**. Furthermore, the NMR spectra of the three compounds were closely related. The main difference between the ^{13}C NMR spectra of compounds **3** and **4** was the absence in the latter spectrum of the signals due to a carbonyl at C-24 and a methyne at C-25, appearing in turn two signals at $\delta = 64.7$ (d) and 58.1 (s) attributable to the carbon atoms of an epoxide group as that possessed by compound **2**. In fact, the signal at $\delta = 64.7$ was correlated in the HSQC spectrum with a proton signal at 2.68 (dd, 1H, $J = 6.7$ and 5.8 Hz), while in the HMBC spectrum showed cross peaks with the signals at 1.30 (s, 3H) and 1.25 (3H, s) due to the methyl groups at C-26 and C-27. All these data confirmed the presence of an epoxide ring at C-24,C-25, and therefore it was proposed the structure 24,25-epoxy-12β-acetoxycholest-4-en-3-one for compound **4**.

The ^{1}H NMR spectrum of compound **5** displayed four signals attributable to the methyl groups of a cholestane at $\delta = 1.29$ (s, 6H, H-26/H-27), 1.18 (s, 3H, H-19), 1.02 (d, 3H, $J = 6.8$ Hz, H-21) and 0.72 (s, 3H, H-18). However, taking into account the molecular formula $C_{26}H_{40}O_2$, established by HRMS, it was concluded that compound **5** was a nor-cholestane derivative. A comparison of the NMR data of compound **5** with those of compounds **1**–**4** revealed that compound **5** also possessed the previously described C-19 tetracyclic nucleus containing an α,β-unsaturated ketone in ring A. Therefore, the side chain had to be formed by seven carbons, three of them corresponding to the methyl groups that gave rise to the ^{1}H NMR signals above mentioned at 1.02 (d, 3H, $J = 6.8$ Hz, H-21) and 1.29 (s, 6H, H-26/H-27). Furthermore, this latter signal together with the ^{13}C NMR singlet at $\delta = 70.6$ ppm were consistent with the presence at C-25 of a hydroxyl group whose IR absorption was observed at 3445 cm^{-1}. Finally, the ^{1}H NMR spectrum showed two olefinic proton signals at $\delta = 5.52$ (d, 1H, $J =$ 15.7) 5.43 (dd, 1H, $J = 15.7$ and 8.4 Hz) that were assigned to the protons of a *trans*-disubstituted double bond. Since in the COSY spectrum the doublet due to the Me-21 group at $\delta = 1.02$ showed a cross peak with a proton signal at $\delta = 2.05$ (m, 1H, H-20) which in turn was correlated with the olefinic proton signal at $\delta = 5.43$, it was determined the localization of the double bond at C-22,C-23. All these data led to propose for compound **5** the structure (22*E*)-25-hydroxy-24-nor-cholest-4,22-dien-3-one.

Compound **6** was isolated as a solid whose molecular formula, $C_{29}H_{46}O_4$, deduced from the HRMS measurement, together with a general inspection of the ^{1}H and ^{13}C NMR spectra indicated that it was an acetylated cholestane derivative.

In addition to those corresponding to the acetoxyl group, the most distinctive signals of the ^{13}C NMR spectrum were a carbonyl signal at $\delta = 203.4$ (s), assigned to a ketone conjugated with a trisubstituted double bond that gave rise to the carbon signals at $\delta = 150.9$ (s) and 126.0 (d), and two signals at $\delta = 71.1$ (s) and 65.3 (d) assigned to two carbons bearing oxygenated functions. One of these had to be the acetoxyl group, while the other one was identified as a hydroxyl group that gave rise to the IR absorption at 3480 cm^{-1}. The location of these functional groups on the cholestane skeleton was performed through the interpretation of the COSY, HSQC and HMBC spectra.

Table 3
Growth inhibition on human tumor cell lines shown by compounds **1**–**8** (GI_{50} values in μg/mL)

Compound	A	B	C	D	E	F	G	H	I	J	K	L
1		2.0		3.2	4.2		1.1	3.9	3.3	3.6	4.0	4.5
2		1.7	5.0	2.2	3.1		1.2	3.1	2.6	2.9	3.1	3.4
3	4.2	1.6	4.4	3.5	3.6	4.9	1.6	3.3	4.3	2.0	1.5	3.4
4	4.1	1.5	5.2	3.4	3.4	4.4	1.3	2.9	3.5	2.2	1.9	3.6
5		1.4		3.3	4.9		0.9	3.7	5.0	3.5	4.4	
6	2.6	1.6	2.1	3.0	1.5	3.8	1.4	2.1	2.2	2.1	2.9	2.6
7	2.3	2.3	2.2	3.2	2.0	4.2	1.5	2.4	2.9	2.0	1.7	2.9
8		3.0					1.8					

A: DU-145; B: LN-caP; C: IGROV; D: SK-BR3; E: SK-MEL-28; F: A549; G: K-562; H: PANC1; I: HT29; J: LOVO; K: LOVO-DOX; and L: HELA.

Thus, the carbon signal at $\delta = 126.0$, that was correlated in the HSQC spectrum with a signal at $\delta = 6.22$ (d, 1H, $J =$ 4.6 Hz), was assigned to the olefinic methyne in β position with respect to the carbonyl group. The signal at $\delta = 150.9$, due to the olefinic carbon α to the carbonyl group, showed in the HMBC spectrum three-bonds correlations with the proton signal at $\delta = 0.95$ (s, 3H), corresponding to the methyl group at C-19, and with a proton signal $\delta = 2.57$ (dd, 1H, $J = 16.0$, 4.4 Hz) that, together with a signal at $\delta = 1.96$ (dd, 1H, $J = 16.0$, and 12.4 Hz), were assigned to a methylene adjacent to the carbonyl group. Taking into account the cholestane framework, this correlations were consistent with the location of the trisubstituted double bond at C-4,C-5, in ring A, and the ketone carbonyl at C-6, in ring B. On the other hand, in the HSQC spectrum the carbon signal at $\delta = 65.3$ (d) showed a cross peak with a proton signal at $\delta = 5.27$ (m, 1H), that was assigned to a proton geminal to the acetoxyl group. In the COSY spectrum, this proton signal was correlated with the H-4 olefinic proton signal at $\delta = 6.22$, thus defining the location of the acetoxyl group at C-3. At this point, the tertiary hydroxyl group had to be located at C-25 [$\delta_C = 71.1$ (s)] in agreement with the signal of the ^{1}H NMR spectrum at $\delta = 1.22$ (s, 6H), corresponding to the methyl groups at C-26 and C-27.

Finally, the correlations observed in the NOESY spectrum were consistent with the usual stereochemistry of the hydrocarbon framework of cholestanes, while the multiplicity displayed by the signal of H-3 at $\delta = 5.27$ (m, $W_{1/2} = 11.8$ Hz), typical of an equatorial proton [25], indicated an axial or α orientation of the acetoxyl group. All the data were in agreement with the structure 3α-acetoxy-25-hydroxycholest-4-en-6-one for compound **6**.

The NMR spectra of compound **7** were closely similar to those of compound **6** above described, except by the presence of the signals corresponding to an additional acetoxyl group. In fact, a detailed comparison with the NMR data of compound **6** led to determine that compound **7** was a cholestane which possessed the same γ-acetoxy-α,β-unsaturated ketone moiety in rings A/B and side chain that compound **6**. Therefore, the additional acetoxyl group had to be located in the tetracyclic nucleus and linked to a methyne whose signals were observed at $\delta_H = 5.28$ (ddd, 1H, $J = 10.5$, 10.5, and 5.3 Hz) and $\delta_C = 70.6$ (d). In the COSY spectrum the signal of the proton geminal to the acetoxyl group ($\delta_H = 5.28$) was correlated with a methyne proton signal at $\delta = 1.73$ (t, 1H, $J = 10.5$ Hz, H-9) and with the signal of a methylene at $\delta = 2.37$ (dd, 1H, $J = 12.2$, 5.3 Hz, H-12) and 1.25 ppm (m, 1H, H-12). On the other hand, in the HMBC spectrum, the proton signal at $\delta = 5.28$ showed a cross peak with a quaternary carbon signal at $\delta = 39.8$ (s, C-10) which in turn was correlated with the proton signal at $\delta = 1.07$ (s, 3H) due to the methyl at C-19. These correlations indicated that the acetoxyl group was located at C-11. Furthermore, an axial orientation for H-11 was deduced from the coupling constants $^3J_{9,11ax} = 10.5$ Hz, $^3J_{11ax,12ax} = 10.5$ Hz, and $^3J_{11ax,12eq} = 5.3$ Hz. Based on these spectroscopic evidences it was proposed for compound **7** the structure 3α,11α-diacetoxy-25-hydroxycholest-4-en-6-one.

All the steroids isolated from *D. acanthina* were tested in bioassays directed to detect in vitro activity against a panel of 12 human tumor cell lines (see Section 2). The measured parameters were GI_{50} (concentration that causes 50% growth inhibition), TCI (concentration that causes total

Table 4
Cytostatic effect on human tumor cell lines shown by compounds **1**–**8** (TGI values in μg/mL)

Compound	A	B	C	D	E	F	G	H	I	J	K	L
1		4.1					2.8					
2		3.8		6.8			2.9		8.5	9.6		
3		3.7					3.8		9.0	5.0		
4		4.0					3.7			8.1	7.4	
5		3.3					3.2					
6	8.9	5.3	4.9	9.5	3.4		4.3	6.2	4.4	6.1	8.0	6.9
7	4.1	4.7	4.2	8.6	4.0	9.9	3.7	4.8	5.9	4.5	4.8	7.4
8		7.0					4.1					

A: DU-145; B: LN-caP; C: IGROV; D: SK-BR3; E: SK-MEL-28; F: A549; G: K-562; H: PANC1; I: HT29; J: LOVO; K: LOVO-DOX; and L: HELA.

Table 5
Cytotoxic effect on human tumor cell lines shown by compounds **1**–**8** (LC_{50} values in μg/mL)

Compound	A	B	C	E	G	H	I	J
1		8.5			6.8			
2		8.1			7.2			
3		8.5			9.0			
5		7.8						
6				7.3			9.0	
7	7.3	9.3	8.0	7.8	9.1	9.6		9.9
8					9.5			

A: DU-145; B: LN-caP; C: IGROV; E: SK-MEL-28; G: K-562; H: PANC1; I: HT29; and J: LOVO.

growth inhibition: cytostatic effect) and LC_{50} (concentration that causes 50% cell killing: cytotoxic effect).

The most significant GI_{50} values (<5 μg/mL) are presented in Table 3. In general, all compounds showed activity as growth inhibitors of a number of cell lines. The higher levels of activity were observed against the cell lines LN-caP and K-562, with most of the compounds displaying GI_{50} values lower than 2 μg/mL. In addition, the tested steroids showed the cytostatic effects shown in Table 4, with all the compounds being active against the cell lines LN-caP and K-562. Compounds **6** and **7** presented the broader range of activity with significant cytostatic effects on most of the cell lines. Finally, cytotoxic effects were observed on a reduced number of cell lines, as shown in Table 5. All the compounds resulted cytotoxic against one or two cell lines excepting compound **7** that was active against seven of the assayed cell lines.

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