Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 3171–3177

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Cytotoxic Activity of Novel C7-Functionalized Spongiane Diterpenes

Manuel Arnó,[a] Liliana Betancur-Galvis,[b,*] Miguel A. González,[a,*] Jelver Sierra[b] and Ramón J. Zaragozá[a]

[a]*Departamento de Química Orgánica, Universidad de Valencia, E-46100 Burjassot, Valencia, Spain*
[b]*Grupo Infección y Cáncer, Universidad de Antioquia, A.A1226 Medellín, Colombia*

Received 31 January 2003; accepted 1 April 2003

Abstract—Based on two lead cytotoxic spongiane diterpenes, a new series of C7-oxygenated derivatives were synthesized and evaluated for their antitumor activity in vitro against the cancer cell lines HeLa and HEp-2. In general, introduction of either hydroxyl or acetoxy groups at C-7 did not improve the resultant cytotoxicity, while the presence of a butyrate ester led to more active compounds (CC_{50} = 4.0–9.5 μM).

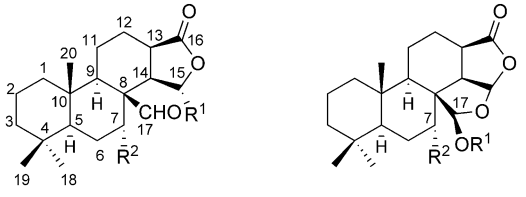
Introduction

Spongiane diterpenoids are common metabolites found in marine organisms such as sponges and shell-less mollusks (nudibranchs). Nudibranchs are often conspicuously colored animals containing diet-derived natural products taken from the sponges.[1] These compounds are generally used in a defensive basis against predation and some of them have shown an interesting and wide profile of biological activity.[2] Despite the diverse biological properties of this family of compounds, little effort has been made for their preparation and biological study, specially of structural analogues.

As a part of our research program towards the synthesis of bioactive terpene compounds starting from chiral building blocks such as podocarpenone **13**, prepared from (−)-abietic acid, and carvone, we have developed several routes leading to a variety of enantiopure spongiane-type diterpenes,[3] and recently, we reported the synthesis of (−)-spongian-16-oxo-17-al (**1**) and (−)-acetyldendrillol-1 (**12**).[4] Then, we started a line of investigation in order to study the biological activity of most of the synthetic spongianes obtained by our group, as well as some derivatives and synthetic intermediates.

For example, the antiherpetic activity of these compounds was studied against herpes simplex virus type 2 (HSV-2), but this was weak. However, several compounds exhibited significant cytotoxic activity against human cervix epitheloid carcinoma (HeLa) and human larynx epidermoid carcinoma (HEp-2) cancer cell lines (CC_{50} 3.5–37.5 μg/mL in 48 h assays).[5] These results prompted us to further study this structural class of molecules. Consequently, two leads for this family of compounds were selected, in particular, one for tetracyclic spongianes (**1**, spongian-16-oxo-17-al) and another for pentacyclic spongianes (**7**, dendrillol-1).

A new series of C7-oxygenated derivatives was then designed and synthesized to determine their antitumor activity upon introduction/modification of a functional



1 R^1= R^2= H
2 R^1= OAc; R^2= H
3 R^1= H; R^2= OH
4 R^1= H; R^2= OAc
5 R^1= OAc; R^2= OAc
6 R^1= OAc; R^2= OCOPr

17 R^1= R^2= H
18 R^1= H; R^2= OH
19 R^1= H; R^2= OAc
10 R^1= H; R^2= OCOPr
11 R^1= OAc; R^2= OAc
12 R^1= OAc; R^2= H

Figure 1. Chemical structures of tested spongianes.

*Corresponding authors. M.A. González tel.: +34-9635-44327; fax: +34-9635-44328; L. Betancur-Galvis tel.: +57-4510-6059; fax: +57-4510-6062. E-mail: miguel.a.gonzalez@uv.es (M.A. González, chemical aspects), labeta@catios.udea.edu.co (L. Betancur-Galvis, biological aspects).

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doi:10.1016/S0968-0896(03)00230-X

group at C-7 (Fig. 1, Scheme 3). In this paper, we report on the synthesis and antitumor activity of several new 7-oxygenated spongianes such as tetracyclic compounds **3**–**6** and pentacyclic spongiane **11**. The known C7-oxygenated aplyroseols **8**–**10** as well as the tricyclic intermediates **20** and **21** (1:1 mixture of anomers) were also tested for their cytotoxicity.

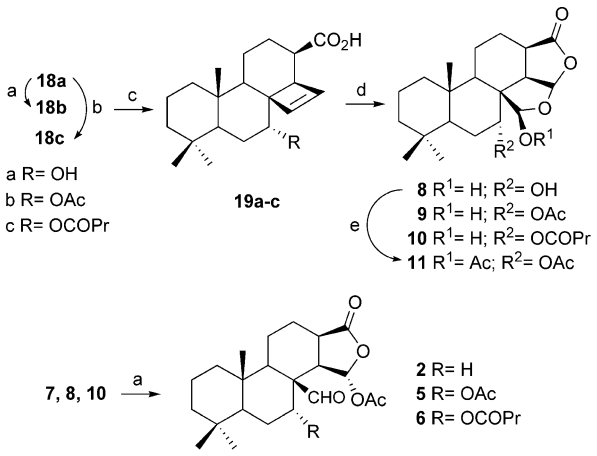
Results and Discussion

Chemistry

The synthesis of the C7-functionalized spongianes used in this work begins with the preparation of the required hydroxy-cyclobutene methyl ester **18a** in eight steps starting from podocarpenone (+)-**13**, following our established procedure (Scheme 1).[6] Thus, podocarpenone **13** was hydroxylated at C-7 by oxidation with *m*-CPBA of its corresponding 7,13-dienyl acetate (**14**) to give hydroxy enone **15** in 72%. Irradiation of enone **15** in dry acetone saturated with acetylene at −30 °C gave stereoselectively photoadduct **16** in 63% yield. Homologation at C-13 of **16** was carried out by cyanophosphorylation with $(EtO)_2P(O)CN$ and LiCN, followed by removal of the phosphate moiety using SmI_2 and *t*-BuOH. This protocol afforded a 3:7 (β/α) mixture of nitriles **17** in 83% yield. Alkaline hydrolysis of both nitriles with aqueous KOH in refluxing ethylene glycol ethyl ether and subsequent esterification with diazomethane and equilibration with sodium methoxide at 80 °C gave compound **18a** in 58% yield. Cyclobutene-ester **18a** can be elaborated in two separate approaches to obtain either pentacyclic or tetracyclic spongiane diterpenes. This will depend on the functional group present at C-13 during the cleavage of the cyclobutene ring with ozone.

In the first approach (Scheme 2), the alcohol **18a** was converted into the ester derivatives **18b**–**c** by esterification with acetic anhydride and butyric anhydride, respectively.

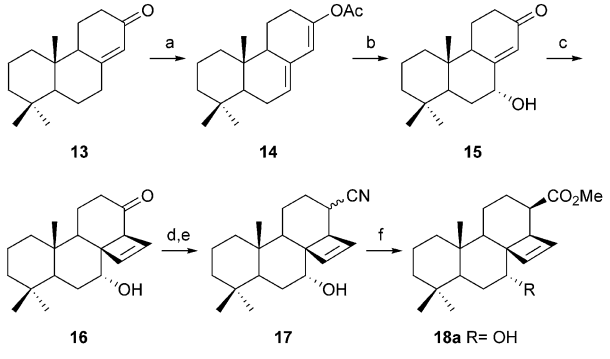
Selective saponification of the methyl ester moiety of compounds **18a**–**c** gave the acids **19a**–**c**, which after



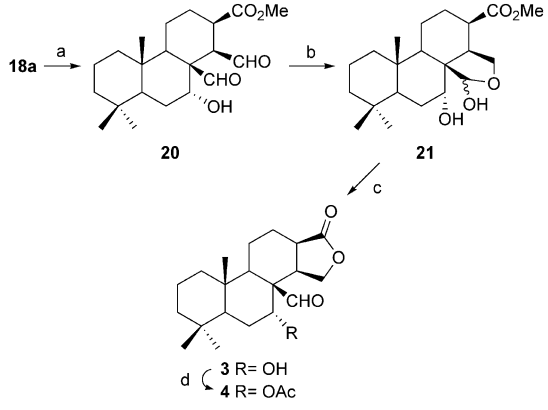
Scheme 2. Reagents and conditions: (a) Ac_2O, 4-pyrrolidinopyridine (4-PP), Et_3N; (b) butyric anhydride, 4-PP, Et_3N; (c) KOH in aq MeOH; (d) (1) O_3, DCM; (2) Me_2S; (e) Ac_2O–AcOH, 1% H_2SO_4.

ozonolysis of the cyclobutene ring led to the formation of pentacyclic compounds (**8**–**10**) by concomitant lactone–hemiacetal formation.[6] The aplyroseols **8** and **10** as well as dendrillol-1[7] (**7**) were derivatized via an optimized hemiacetal-ring opening under basic acetylation conditions[8] leading to the tetracyclic compounds (**2**, **5**, and **6**) bearing an α-acetoxy group at C-15. The 15α-acetoxy orientation in these analogues was deduced from their corresponding ^1H NMR and NOE difference spectra. As H-15 resonated as a singlet, only a H-15β orientation can explain a zero coupling constant due to its ∼90° dihedral angle with H-14. This was confirmed by irradiation of the H-15β signal, which caused NOE enhancements of both H-7β and H-17 signals. Acetylation of deacetylaplyroseol-2 (**8**) to obtain diacetate **11** was carried out as reported in our synthesis of acetyldendrillol-1.[4]

On the other approach, as outlined in Scheme 3, an analogous sequence of steps to that developed for the synthesis of **1** led to tetracyclic spongianal **3**. Thus, ozonolysis of the cyclobutene ring in ester **18a** occurs without lactonization to give dialdehyde **20** in essentially quantitative yield, which was used without further purification in view of its high tendency for lactone–



Scheme 1. Reagents and conditions: (a) Ac_2O/AcCl, py, 97%; (b) *m*-CPA, then $Na_2S_2O_2$–$NaHCO_3$, 74%; (c) C_2H_2, acetone, hv, 63%; (d) $(EtO)_2P(O)CN$, LiCN; (e) SmI_2/*t*-BuOH, 83%, two steps; (f) (1) KOH, $HO(CH_2)_2OEt$; (2) CH_2N_2; (3) NaOMe, MeOH, 58%, three steps.



Scheme 3. Reagents and conditions: (a) (1) O_3, DCM; (2) Me_2S, 99%; (b) $NaBH_4$, MeOH, 99%; (c) TFA, 2:3 acetone–H_2O, 47%; (d) Ac_2O, 4-PP, Et_3N, 70%.

hemiacetal formation. Reduction of **20** using $NaBH_4$/MeOH at 0 °C afforded a 1:1 epimeric mixture at C-17 of hemiacetals **21** in high yield. Lactonization of hemiacetals **21** using TFA in a 2:3 (v/v) mixture of acetone/water at reflux produced 7α-hydroxyspongian-16-oxo-17-al (**3**) in moderate yield (47%), which was then acylated to give acetate **4** in 70% yield.

Antitumor evaluation

The spongianes **1**–**12** and compounds **20**–**21** were tested in vitro for potential cytotoxic activities determining the cytotoxic concentration needed to detach 100% (CC_{100}) of the cell monolayer on two cancer lines (HeLa and HEp-2), the Vero cell line and the BHK primary cell culture (Table 1).

Table 1. In vitro activity of Spongiane diterpenes against cell growth expressed as CC_{100} (μg/mL)[a]

Compd	Cell lines[b]			
	HeLa	HEp-2	Vero	BHK
1	20	20	20	>40
2	10	10	10	20
3	>40	>40	>40	>40
4	>40	>40	>40	>40
5	10	10	10	20
6	10	10	10	20
7	10	10	10	20
8	10	20	10	20
9	10	10	10	10
10	10	5	10	10
11	40	40	40	40
12	>40	>40	>40	>40
20	15	15	15	30
21	40	40	40	>40

[a]Minimal toxic dose that detached 100% of the cell monolayer in 48 h.
[b]HeLa, human cervix epitheloid carcinoma ATCC CCL-2; HEp-2, human larynx epidermoid carcinoma ATCC CCL-23; Vero, *Cercopithecus aethiops* African green monkey kidney ATCC CCL-81; BHK, *Mesocricetus auratus*, golden hamster kidney ATCC CCL-10.

In the series of tetracyclic spongianes (**1**–**6**), introduction of either α-hydroxyl or α-acetoxy groups at C-7 in **1** showed a 2-fold reduction in activity in both cancer cell lines. Similarly, hemiacetals **21** were less active than the corresponding 7-deoxy-derivatives.[5] The most cytotoxic tetracyclic compounds were the 15-acetoxy analogues (**2**, **5** and **6**), which clearly shows that the 15α-acetoxy group enhances the resultant cytotoxicity for the cell lines tested. On the other hand, the cytotoxicity data in the series of pentacyclic spongianes (**7**–**12**) indicated a comparable activity, independently of the substituent at C-7, against the HeLa cell line. For the HEp-2 cell line, however, the presence of a C-7 hydroxyl group (**8**, deacetylaplyroseol-2) led to a significant reduction of activity while a butyrate ester (**10**, aplyroseol-1) increased 2-fold the activity. These activity data also suggested the importance of the lactone–hemiacetal system for cytotoxicity, since esterification of the C-17 hydroxyl group (compounds **11** and **12**) caused a dramatic reduction in activity in all the cell lines tested. To establish accurate values for cytotoxicity, concentrations required to reduce cell viability by 50% [50% cytotoxic concentration (CC_{50})] for compounds that showed CC_{100} of 10 μg/mL or less were obtained using the tetrazolium-dye (MTT) method.[9] The incubation period for these assays was 48 h (Table 2).

Table 2. Cytotoxic activity of spongiane diterpenes determined by the MTT technique expressed as CC_{50} (μg/mL)[a]

Compd	Cell lines[b]			
	HeLa	HEp-2	Vero	BHK
2	7.3±0.8	4.2±0.1	10.6±1.8	9.7±0.8
5	6.4±0.6	4.1±0.4	5.4±0.8	9.1±0.4
6	4.2±0.4	3.9±0.3	5.2±0.8	6.9±1.3
7	14.8±1.1	3.5±0.6	4.7±0.5	8.0±0.6
8	9.4±1.1	6.9±1.0	4.9±1.1	7.1±0.3
9	4.6±0.2	4.0±0.5	9.0±0.3	9.5±1.9
10	3.6±0.4	2.6±0.3	4.6±0.8	4.0±0.5
Vincristine	0.05±0.01	0.05±0.01	1.1±0.2	0.61±0.01

[a]50% cytotoxic concentration in 48 h.
[b]HeLa, human cervix epitheloid carcinoma ATCC CCL-2; HEp-2, human larynx epidermoid carcinoma ATCC CCL-23; Vero, *C. aethiops* African green monkey kidney ATCC CCL-81; BHK, *M. auratus*, golden hamster kidney ATCC CCL-10.

As expected from the preliminary screening, compounds having a C-7 butyrate ester were the most potent in both tetracyclic (**6**)[10] and pentacyclic (**10**) series, which confirms that the C-7 butyrate ester is partly contributing toward cytotoxicity in the spongiane skeleton. On comparing the activities of **2** and **5**, it can be concluded that the introduction of the 7α-acetoxy group does not change the cytotoxicity in **2**. With respect to active pentacyclic spongianes, the data indicated that while introduction of a 7α-hydroxyl group (compound **8**) in **7** reduces the resultant cytotoxicity for HEp-2 cells, the presence of a butyrate ester (**10**, aplyroseol-1) enhances its activity, specially against HEp-2 cells. In order to make our results comparable to other observations, we have also determined the CC_{50} values for **6** and **10** in a more standard assay of 72 h (see Table 3).

Compound **6** was the most cytotoxic tetracyclic spongiane tested, with CC_{50} of 2.4, 2.1 μg/mL on HeLa and HEp-2, respectively. Interestingly, the CC_{50} value on HeLa cells of **6** is quite significant due to their known resistance to chemotherapy. The most cytotoxic compound tested was **10** with CC_{50} of 1.7 μg/mL on HEp-2. Aplyroseol-1 (**10**) has previously shown mild cytotoxicity against other tumor cells, ED_{50} on PS cells (in vitro

Table 3. Cytotoxic activity of spongiane diterpenes determined by the MTT technique expressed as CC_{50} (μg/mL)[a]

Compd	Cell lines[b]			
	HeLa	HEp-2	Vero	BHK
6	2.4±0.1	2.1±0.1	3.5±0.1	3.3±0.7
10	4.0±0.2	1.7±0.1	4.5±0.5	3.2±0.3

[a]50% cytotoxic concentration in 72 h.
[b]HeLa, human cervix epitheloid carcinoma ATCC CCL-2; HEp-2, human larynx epidermoid carcinoma ATCC CCL-23; Vero, *C. aethiops* African green monkey kidney ATCC CCL-81; BHK, *M. auratus*, golden hamster kidney ATCC CCL-10.

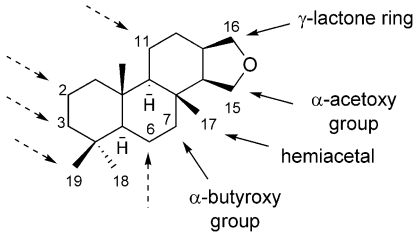


Figure 2. SAR in the spongiane skeleton: ⟶ already studied for cytotoxicity. - - - ➝ of interest to be studied.

lymphocytic leukaemia) of 6.0 μg/mL.[11] Significant cytotoxicity in vitro has also been described for aplyroseol-2 (**9**) against murine lymphoma L1210 and human epidermoid carcinoma KB cells 1.9 and 2.5 μg/mL, respectively.[2d] These results are consistent with our previous report[5] that introduction of an α-acetoxy group at C-15 enhances the cytotoxic activity in tetracyclic spongianals while modification of the hemiacetal ring moiety in pentacyclic spongianes causes a complete loss of activity (Fig. 2).

Conclusions

In conclusion, we have prepared and tested several spongianes for their antitumor activity in vitro. In general, the cytotoxicity shown was in the range 10–50 μM concentration for 48 h assays. Two compounds (**6** and **10**) exhibited important cytotoxicity (~5 μM in 72 h assays) but little selectivity for tumor cells. From our results, it can be deduced that molecules having a γ-acetoxybutanolide moiety, as the known cytotoxic agent acetomycin and related analogues,[12] are good candidates in the process of drug design. In addition, we have shown further evidence that the lactone–hemiacetal system present in pentacyclic spongianes is actually an important pharmacophore unit, which has very few examples outside the chemistry of marine organisms. The structural features responsible of the biological activity here described provide a good reference for further chemotherapeutic and mechanistic studies, which are currently being developed.[13]

Experimental

Chemistry

Melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were determined using a 5 cm path-length cell, using chloroform as solvent (concentration expressed in g/100 mL). $[\alpha]_D$ values are given in $10^{-1\circ}$ cm^2 g^{-1}. Infrared (IR) spectra were taken as KBr pellets, liquid films on NaCl plates or solutions in chloroform. NMR spectra were recorded on 300 or 400 MHz spectrometers with tetramethylsilane as an internal standard. All spectra were recorded in $CDCl_3$ as solvent unless otherwise described. Complete assignments of ^{13}C NMR multiplicities were made on the basis of DEPT experiments. HMQC and NOE experiments were used in some ^{1}H NMR assignments. *J* values are given in Hz. In all compounds, NMR assignments are given with respect to the numbering in spongiane skeleton. Mass spectra (MS) were run by electron impact (EI) at 70 eV. Reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F-254 in 0.25 mm thick plates. Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a 10% sulfuric acid solution and heating on a hotplate. Purifications were performed by flash chromatography on Merck silica gel (230–400 mesh). All non-aqueous reactions were carried out in an argon atmosphere in oven-dried glassware. Commercial reagent grade solvents and chemicals were used as received unless otherwise noted. Combined organic extracts were washed with brine, dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. Preparation and spectroscopic data for compounds **1**, **7**–**10**, and **13**–**19** can be found in refs 4, 6 and 7.

General procedure for the preparation of spongianals 2, 5 and 6. Example: (−)-15α-Acetoxyspongian-16-oxo-17-al (2). Acetic anhydride (25 μL, 0.26 mmol) was added to a stirred solution of dendrillol **7** (15.0 mg, 0.045 mmol), 4-pyrrolidinopyridine (4-PP, 98%, 1.0 mg, 0.007 mmol) in dry Et_3N (0.4 mL) at 0 °C. The reaction mixture was then allowed to warm to room temperature, stirred for 70 min, and diluted with diethyl ether. The organic phase was washed with 1.5 M HCl and brine, dried, filtered and concentrated. Purification of the residue by flash chromatography, using hexane–ethyl acetate (from 7:3 to 6:4) as eluent, provided spongiane **2** (15.2 mg, 90%) as a white solid: mp 161–162 °C (from hexane–EtOAc); $[\alpha]_D^{23}$ −9.0 (*c* 0.69); IR (KBr) 2943, 2739, 1790, 1769, 1711, 1214 cm^{-1}; ^{1}H NMR (300 MHz) δ 9.93 (1H, d, *J* = 1.3, H-17), 6.18 (1H, s, H-15β), 2.97 (1H, dd, *J* = 7.9, 7.7, H-13), 2.72 (1H, ddd, *J* = 13.1, 3.2, 3.2, H-7β), 2.51 (1H, br d, *J* = 12.1, H-12β), 2.26 (1H, d, *J* = 7.9, H-14), 2.08 (3H, s, OCO*Me*), 0.85, 0.76 and 0.68 (3H each, each s, H-18, H-19 and H-20); ^{13}C NMR (75 MHz) δ_C 204.2 (d), 176.3 (s), 168.5 (s), 93.9 (d), 56.4 (d), 55.7 (d), 53.3 (d), 49.4 (s), 41.7 (t), 39.0 (t), 37.8 (s), 35.8 (t), 35.1 (d), 33.3 (q), 33.2 (s), 22.4 (t), 21.4 (q), 20.9 (q), 18.7 (t), 18.5 (t), 16.1 (t), 15.1 (q); HRMS (EI) *m*/*z* 376.2249 $[M]^+$, calcd for $C_{22}H_{32}O_5$: 376.2250.

The following spongianals were prepared accordingly.

7α,15α-Diacetoxyspongian-16-oxo-17-al (5). The treatment of deacetoxyaplyroseol-2 (**8**) (10 mg, 0.03 mmol) provided spongiane **5** (10.7 mg) containing a 20% of two pentacyclic diacetates. This mixture was further repurified by chromatography eluting with hexane–ethyl acetate (from 9:1 to 8:2) to give 4.0 mg of pure aldehyde **5** as a colorless oil: $[\alpha]_D^{18}$ −60.6 (*c* 0.33); IR (neat/NaCl) 2925, 1798, 1743, 1372, 1242, 1046 cm^{-1}; ^{1}H NMR (300 MHz) δ 9.93 (1H, s, H-17), 6.12 (1H, s, H-15β), 5.62 (1H, dd, *J* = 2.6, 2.6, H-7β), 3.04 (1H, dd, *J* = 7.8, 7.8, H-13), 2.70 (1H, d, *J* = 8.1), 2.51 (1H, br d, *J* = 12.0), 2.10 (3H, s, OCO*Me*), 2.05 (3H, s, OCO*Me*), 0.78, 0.73 and 0.71 (3H each, each s, H-18, H-19 and H-20); ^{13}C NMR (75 MHz) δ_C 202.6 (d), 176.4 (s), 169.6

(s), 168.3 (s), 93.9 (d), 69.2 (d), 52.0 (d), 51.1 (s), 47.5 (d), 45.2 (d), 41.5 (t), 38.8 (t), 37.8 (s), 35.0 (d), 33.0 (q), 32.6 (s), 23.6 (t), 22.0 (t), 21.2 (q), 21.2 (q), 20.7 (q), 18.5 (t), 16.0 (t), 14.5 (q); HRMS (EI) *m/z* 434.2310 $[M]^+$, calcd for $C_{24}H_{34}O_7$: 434.2304.

15α-Acetoxy-7α-butanoyloxy-spongian-16-oxo-17-al (6). Aplyroseol **10** (7.2 mg, 0.018 mmol) gave, after chromatography using 7:3 hexane–ethyl acetate as eluent, a mixture (6.1 mg) of spongiane **6** and two epimeric diacetates at C-17. This mixture was further repurified by chromatography eluting with hexane–ethyl acetate (from 95:5 to 7:3) to give 3.0 mg of aldehyde **6**, containing a 25% of an unidentified pentacyclic diacetate (probably acetylaplyroseol-1), as a colorless oil: ^{1}H NMR (300 MHz) δ 9.93 (1H, s, H-17), 6.15 (1H, s, H-15β), 5.65 (1H, dd, *J* = 2.6, 2.6, H-7β), 3.03 (1H, dd, *J* = 7.7, 7.7, H-13), 2.65 (1H, d, *J* = 8.1), 2.51 (1H, br d, *J* = 12.0), 2.33 (2H, t, *J* = 8.2), 2.03 (3H, s, OCO*Me*), 0.97 (3H, t, *J* = 6.8), 0.77, 0.73 and 0.71 (3H each, each s, H-18, H-19 and H-20); ^{13}C NMR (75 MHz) δ_C 202.7 (d), 176.4 (s), 172.2 (s), 168.1 (s), 93.6 (d), 68.7 (d), 51.9 (s), 51.2 (d), 47.5 (d), 45.2 (d), 41.5 (t), 38.8 (t), 37.8 (s), 36.5 (t), 35.1 (d), 33.0 (q), 32.6 (s), 23.8 (t), 22.0 (t), 21.2 (q), 20.6 (q), 18.6 (t), 18.5 (t), 16.1 (t), 14.5 (q), 13.7 (q).

7α-17β-Diacetoxy-15,17-oxidospongian-16-one (acetylaplyroseol-2) (11). Deacetoxyaplyroseol-2 (**8**) (5 mg, 0.015 mmol) was dissolved in a 1% H_2SO_4 solution in acetic acid (1.0 mL), acetic anhydride (42 μL, 0.44 mmol) was added and the resulting mixture was heated at 65 °C for 17 h. The brownish mixture was cooled to rt, poured into water, and extracted with diethyl ether. The combined organic layers were washed with 10% aqueous $NaHCO_3$ (carefully) and brine, dried, filtered, and concentrated. The resulting residue was purified by column chromatography (7:3 hexane–EtOAc) to afford 4.0 mg (65%) of acetylaplyroseol-2 (**11**): $[\alpha]_D^{26}$ −85.7 (*c* 1.3); IR (KBr) 2954, 1785, 1760, 1219 cm^{-1}; ^{1}H NMR (400 MHz) δ 6.26 (1H, s, H-17), 6.06 (1H, d, *J* = 6.0, H-15), 4.80 (1H, dd, *J* = 2.5, 2.5, H-7β), 2.93 (1H, dd, *J* = 11.0, 5.4), 2.80 (1H, dd, *J* = 11.5, 6.0), 2.43 (1H, br d, *J* = 11.5), 2.15 (3H, s, OCO*Me*), 2.06 (3H, s, OCO*Me*), 0.79, 0.78 and 0.72 (3H each, each s, H-18, H-19 and H-20); δ_C (75 MHz) 176.4 (s), 170.1 (s), 168.6 (s), 104.2 (d), 100.0 (d), 72.7 (d), 50.1 (s), 49.1 (d), 48.5 (d), 42.1 (d), 41.6 (t), 38.7 (t), 37.8 (s), 37.3 (d), 33.0 (q), 32.7 (s), 24.3 (t), 23.1 (t), 21.5 (q), 21.3 (q), 21.1 (q), 18.6 (t), 16.1 (t), 14.8 (q); HRMS (EI) *m/z* 434.2312 $[M]^+$, calcd for $C_{24}H_{34}O_7$: 434.2304.

Methyl 7α-hydroxy-8β,14β-dioxopodocarpan-13β-oate (20). Cyclobutene **18a** (57 mg, 0.171 mmol) in CH_2Cl_2 (10.5 mL) was cooled to −78 °C, and ozone was passed into the reaction mixture until a light blue color was observed (15 min). Argon was then bubbled through the solution to remove excess ozone. Dimethyl sulfide (2 mL) was added, and the reaction mixture was slowly allowed to warm to 5 °C in a refrigerator over 40 h. The reaction mixture was then diluted with diethyl ether, washed with H_2O and brine, dried, filtered and concentrate in vacuo to give in essentially quantitative yield the crude dialdehyde **20** (62 mg, 99%) as a solid: ^{1}H NMR (300 MHz) δ 9.97 (1H, s), 9.75 (1H, s), 4.75 (1H, br s, H-7β), 3.66 (3H, s, CO_2Me), 3.23 (1H, m), 3.13 (1H, d, *J* = 5.3), 2.41 (1H, m), 0.84, 0.77, and 0.75 (3H each, each s, H-18, H-19 and H-20); ^{13}C NMR (75 MHz) δ_C 204.6 (d), 201.5 (d), 174.0 (s), 66.6 (d), 54.0 (d), 53.4 (s), 52.0 (q), 52.0 (d), 46.6 (d), 41.6 (t), 39.8 (d), 38.6 (t), 37.9 (s), 32.9 (q), 32.6 (s), 27.7 (t), 27.1 (t), 21.3 (q), 18.5 (t), 16.8 (t), 15.3 (q). The dialdehyde **20** was used directly without further purification due to its tendency to lactone–hemiacetal formation.

Methyl 15,17-epoxy-7α,17-dihydroxy-ent-isocopalan-16-oate (21). To a stirred solution of crude dialdehyde **20** (30 mg, 0.082 mmol) in MeOH (3.4 mL) at 0 °C, $NaBH_4$ (98%, 25 mg, 0.63 mmol) was added. The reaction mixture was stirred for 40 min and then diluted with diethyl ether. The resulting solution was washed with 1.5 M aqueous HCl, brine, dried, filtered and concentrated to yield a crude 1:1 mixture of hemiacetals **21** (30 mg, 99%) as an amorphous solid: ^{1}H NMR (mixture, 300 MHz) δ 5.69 and 5.14 (1H, s, H-17), 3.66 and 3.65 (3H, s, CO_2Me), 0.94, 0.92, 0.85×2, and 0.82×2 (3H each, each s, H-18, H-19 and H-20); ^{13}C NMR (mixture, 75 MHz) δ_C 176.1 (s), 175.9 (s), 102.7 (d), 99.0 (d), 71.8 (d), 69.0 (d), 66.8 (t), 66.5 (t), 53.7 (s), 53.4 (s), 51.6 (q), 51.5 (q), 47.8 (d), 47.5 (d), 44.1 (d), 43.5 (d), 42.8 (d), 41.9 (t), 41.8 (t), 39.5 (d), 39.1 (d), 39.0 (t), 38.5 (t), 38.3 (s), 38.1 (s), 33.3 (q), 33.3 (q), 32.6×2 (s), 30.3 (d), 28.1 (t), 27.8 (t), 21.6 (q), 21.6 (q), 19.6 (t), 19.2 (t), 19.1 (t), 18.6 (t), 18.5 (t), 14.8 (t), 14.6 (q), 14.4 (q). The mixture of epimers at C-17 **21** was used directly in the lactonization reaction.

7α-hydroxy-spongian-16-oxo-17-al (3). To a solution of hemiacetals **21** (27 mg, 0.074 mmol) in a 2:3 mixture of acetone–water (10 mL), TFA (0.7 mL) was added. The resulting mixture was refluxed for 20 h and then diluted with EtOAc and DCM, washed with 10% aq $NaHCO_3$, brine, dried and concentrated. The residue was chromatographed on silica eluting with H–EtOAc (from 8:2 to 5:5) to give γ-lactone **3** (11.6 mg, 47%): $[\alpha]_D^{26}$ −105.0 (*c* 0.8, $CHCl_3$); IR (KBr) 3600–3200, 2949, 1748, 1703 cm^{-1}; ^{1}H NMR (300 MHz) 9.99 (1H, d, *J* = 1.8, H-17), 4.61 (1H, d, *J* = 10.6, H-15), 4.29 (1H, dd, *J* = 10.6, 4.7, H-15′), 3.27 (2H, m), 2.75 (1H, dd, *J* = 6.6, 6.6), 2.69 (1H, m), 2.48 (1H, m), 1.94 (1H, m), 0.89, 0.79 and 0.75 (3H, each s, H-18, H-19 and H-20); δ_C (75 MHz) 207.3 (d), 178.0 (s), 82.9 (d), 69.7 (t), 56.0 (d), 54.3 (s), 54.0 (d), 47.6 (d), 41.5 (t), 38.8 (t), 38.7 (d), 37.7 (s), 33.2 (q), 33.2 (s), 29.6 (t), 23.3 (t), 21.4 (q), 18.5 (t), 16.7 (t), 15.2 (q); HRMS (EI) *m/z* 334.2148 $[M]^+$, calcd for $C_{20}H_{30}O_4$: 334.2144.

7α-acetoxy-spongian-16-oxo-17-al (4). To a suspension of alcohol **3** (4 mg, 0.012 mmol) and 4-pyrrolidinopyridine (small crystal) in Et_3N (0.3 mL) acetic anhydride was added. After being stirred for 2 days the reaction mixture was diluted with EtOAc, washed with 5% aq HCl, brine, dried and concentrated. The residue was purified by column chromatography eluting with H–EtOAc (from 8:2 to 5:5) to furnish acetate **4** (3.1 mg, 70%) as a white solid: $[\alpha]_D^{20}$ −30.0 (*c* 0.1, $CHCl_3$); IR (neat/NaCl) 2924, 2852, 1776, 1735, 1593, 1234, 1113,

1054 cm^{-1}; ^1H NMR (300 MHz) 10.10 (1H, s, H-17), 4.82 (1H, dd, $J=11.7$, 4.8, H-15), 4.20 (2H, m), 2.65 (1H, dd, $J=8.0$, 8.0, H-13), 2.45 (2H, m), 2.12 (3H, s, OCO*Me*), 2.03 (1H, m), 0.91, 0.82 and 0.67 (3H, each s, H-18, H-19 and H-20); δ_C (75 MHz) 203.13 (d), 177.34 (s), 170.17 (s), 82.38 (d), 68.37 (t), 57.17 (d), 54.02 (s), 53.31 (d), 45.52 (d), 41.44 (t), 38.43 (t), 37.44 (s), 37.33 (d), 33.28 (s), 33.12 (q), 25.72 (t), 22.57 (t), 21.44 (q), 21.37 (q), 18.49 (t), 16.72 (q), 15.85 (t); HRMS (EI) *m/z* 376.2254 $[M]^+$, calcd for $C_{22}H_{32}O_5$: 376.2250.

Biological assays

Stock solutions (7 mg/mL) of these compounds for testing in vitro were prepared in dimethyl sulfoxide and stored at 4 °C up to 4 weeks.

Cell culture and virus

The cell lines used were human cervix epitheloid carcinoma cells (HeLa cell line ATCC CCL-2), human larynx epidermoid carcinoma cells (HEp-2 cell line ATCC CCL-23), *C. aethiops* african green monkey kidney cells (Vero cell line ATCC CCL-81), and *M. auratus* Golden hamster kidney (BHK cell line ATCC CCL-10).

All cells were grown in MEM supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 20 mg/mL of L-glutamine, 0.14% $NaHCO_3$, and 1% each of nonessential amino acids and vitamin solution. The cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere.

In vitro assay on cell growth

Cell monolayers were trypsinized and washed with culture medium and then plated at 5×10^3 cells per well for HeLa, HEp-2 and Vero cells and at 2×10^4 cells per well for BHK cells in a 96-well flat-bottomed plate. After 24 h of incubation, each diluted compound was added to the appropriate wells and the plates were incubated for further 48 h at 37 °C in a humidified incubator with 5% CO_2. The cytotoxic activity was expressed as the minimal toxic dose of the compound that induces 100% detachment of the cell monolayer (CC_{100}). The results were obtained by duplicates of at least five dilutions for each of the compounds. The results are expressed as the mean obtained from three different assays.

Cytotoxicity assay

Cells were plated, treated with the compounds and incubated as described above in the assay on cell growth. For the tetrazolium-dye (MTT) cytotoxicity assays,[5] the supernatants were removed from the wells and 28 μL of a MTT (Sigma, 2 mg/mL) solution in phosphate buffered saline (PBS) solution was added to each well. Plates were incubated for 1.5 h at 37 °C, and 130 μL of DMSO was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker for 15 min and absorbency was read at 492 nm on a multiwell spectrophotometer (Titertek Uniskan). Furthermore, all tests were compared with a positive control, Vincristine (sulfate salt), and tested simultaneously under identical conditions as reported previously. The results are obtained from triplicate assays with at least five compound concentrations. The percentage of cytotoxicity is calculated as $[(A-B)/A]\times100$, where A and B are the OD_{492} of untreated and of treated cells, respectively. The results were obtained from three assays with at least five concentrations.

Data analysis

The 50% cytotoxic concentration (CC_{50}) for each compound were obtained from dose–effect curves (not shown).

Acknowledgements

Financial support from the Conselleria de Educación y Ciencia de la Generalidad Valenciana to M.A.G. and Programa Iberoamericano de Ciencia y Tecnologia para el Desarrollo (CYTED) is gratefully acknowledged. We thank Dr. Jerry Pelletier (McGill University, Department of Biochemistry, Montreal, Canada) for performing the protein synthesis inhibition assays.

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