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Cytotoxic Effect (on Tumor Cells) and in Vitro Antiviral Activity against Herpes Simplex Virus of Synthetic Spongiane Diterpenes

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A series of synthetic spongiane-type diterpenes have been tested in vitro for their potential antitumor and antiherpetic activity. Although the antiviral activity of these compounds against herpes simplex virus type 2 (HSV-2) was very weak, some compounds exhibited relevant cytotoxicity in the human tumor cell lines HeLa and HEp-2. The biological activity of formyl spongianes is reported for the first time. With the present study, some structure−activity trends are suggested for the cytotoxic activity of these sponge-derived natural products.

Several spongiane-type diterpenoids showing antifungal, antimicrobial, antifeedant, antiviral, and antitumor activities, as well as PLA_2 inhibition, have been isolated from marine sponges or nudibranchs.[1] Isoagatholactone (**1**)[2] isolated from the sponge *Spongia officinalis* represents the first reported member of the spongiane family. Over the years, different spongiane natural products have been isolated with varying functionalization of ring D and patterns of oxidation on rings A, B, and C.

Among the numerous spongianes possessing a ring D lactone, dorisenone A isolated from the mollusk *Chromodoris obsoleta*[3] has displayed significant cytotoxic activity against different tumor cell lines (IC_{50} of 0.21 and 0.22 μg/mL on murine lymphoma L1210 and human epidermoid carcinoma KB cells, respectively). Within the group of bioactive spongianes having a ring D furan, spongiadiol and its epimer epispongiadiol found in several species of sponges are worthy of mention. These two compounds as well as isospongiadiol isolated from Caribbean *Spongia* species have exhibited antiviral (IC_{50} of 0.25, 12.5, and 2.0 μg/mL against Herpes simplex virus type I, respectively) and antitumor activities (IC_{50} of 0.5, 8.0, and 5.0 μg/mL on P388 murine leukemia cells, respectively).[4] Another interesting group of highly oxygenated, pentacyclic spongianes is typified by the mildly cytotoxic aplyroseol-1 (ED_{50} of 6.5 μg/mL on lymphocytic leukemia PS cells).[5]

This rich diversity of known biological activities prompted us to evaluate several synthetic spongiane diterpenoids as well as some derivatives and precursors with the aim of elucidating structure−activity trends in the spongiane skeleton. In the present work, we describe the in vitro antiherpetic and antitumor activities of a number of synthetic natural spongiane diterpenes and some derivatives including tetracyclic structures with lactone, furan, and hemiacetal groups in ring D, as well as three pentacyclic analogues. It is noteworthy that some of the spongiane diterpenes evaluated here were originally isolated only in quantities that precluded bioactivity studies. Consequently, this study represents the first report on the in vitro biological activity of several of these rare marine secondary metabolites.

Results and Discussion

In the present work we have evaluated thus far the in vitro antitumor and antiviral activity of 14 synthetic compounds (Figure 1) including several naturally occurring spongiane diterpenes (compounds **1**, **4**, **8**, **9**, **12**, and **13**).[6] In addition, we have examined a bioactive 2:1 mixture of compounds **12** and **5**, as well as the 2:1 mixture of *ent*-isocopalane epimers **15**. All these compounds were obtained in enantiomerically pure form from either (+)-podocarp-8(14)-en-13-one[7] or *S*-(+)-carvone.[8] They may be grouped as follows: compounds containing lactone and/or hemiacetal groups such as spongia-12-en-16-one (**1**), 13α-hydroxyspongian-16-one (**2**), 13β-hydroxyspongian-16-one (**3**), spongian-16-oxo-17-al (**4**), 15α-acetoxyspongian-16-oxo-17-al (**5**), 15,16-dideoxy-15,17-oxido-16,17-oxidospongian-16-one (**6**), 15,16-dideoxy-15-hydroxy-16,17-oxidospongian-16-one (**7**), and 15-acetoxy-15,16-dideoxy-16,17-oxidospongian-16-one (aplyroseol-14) (**8**),[9] whose reassigned structure as depicted has recently been confirmed by means of spectral evidence and synthesis of its putative structure;[10] furanoditerpenes such as spongia-13(16),14-diene (**9**), spongia-13(16),14-dien-3-one (**10**), and 3β-methoxy-19-norspongia-13(16),14-diene (**11**);[11] and finally, pentacyclic spongianes such as 17β-hydroxy-15,17-oxidospongian-16-one (dendrillol-1) (**12**), 17β-acetoxy-15,17-oxidospongian-16-one (**13**), and 17β-methoxy-15,17-oxidospongian-16-one (**14**).

A preliminary evaluation of the potential biological activities of all these compounds was carried out using the end-point titration technique (EPTT),[12] in which the cytotoxic activity and the antiviral effect were simultaneously evaluated (Table 1). Vlietinck et al.[12] have reported that only the compounds with reduction factors (R_f) of the viral titer over 1×10^3 show relevant antiviral activity. As can be seen in Table 1, the compounds **3**, **7**, and the 2:1 mixture of **12**/**5** were found to be slightly active against Herpes simplex virus type II (HSV-2), with a R_f of 1×10^1. The furanoditerpenoids **9**−**11** were even less active against HSV-2. For most of these compounds, the nontoxic concentration needed to obtain the largest reduction of the viral titer was approximately the same as the cytotoxic concentration needed to detach 100% (CC_{100}) of the cell monolayer, revealing that the antiviral activity is principally due to cytotoxicity and did not warrant further evaluation by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method.[13] Comparing the antiviral activity against HSV-1 of spongiadiol

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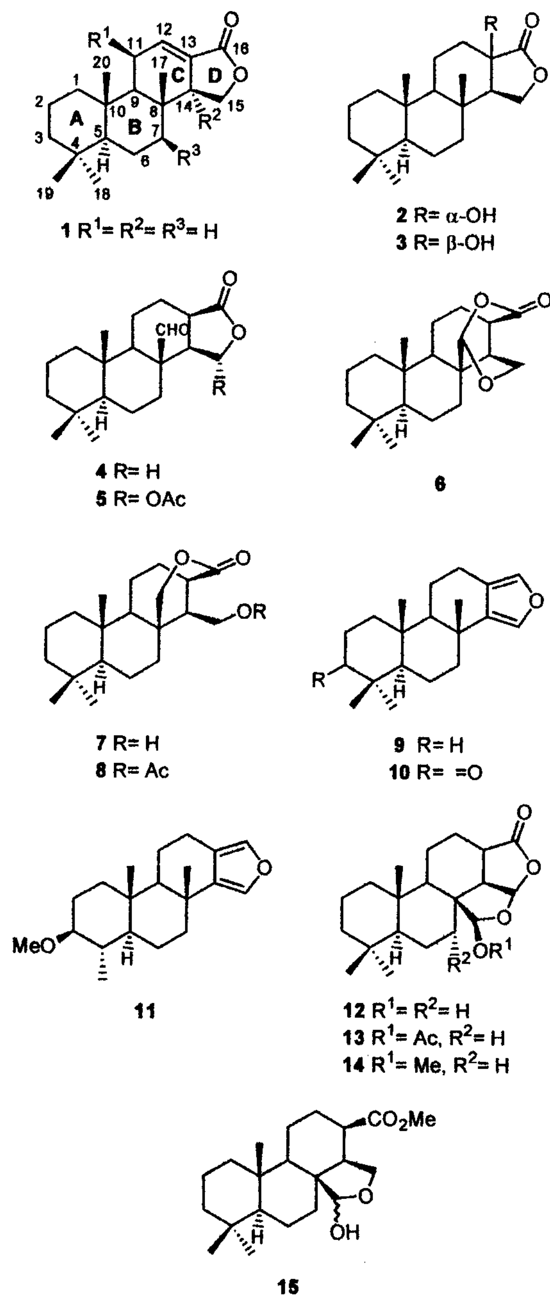

Figure 1.

and analogues reported by McConnell et al.[4] with the weak activity against HSV-2 found for the furanoditerpenoids evaluated in this study suggests that the presence of a β-hydroxymethyl group at C-4 as well as oxygenation in ring A leads to enhanced antiviral activity.

In this preliminary screening, synthetic isoagatholactone **1** showed low cytotoxicity in accordance with the results reported by González et al.[15] Therefore, on the basis of the reports by Higuchi et al.[3] and González et al.,[15] only isoagatholactone derivatives containing β-acetoxy groups at C-11 display relevant antitumor activity.

Compounds with measured CC_{100} values of 30 μg/mL or less against Vero cells using EPTT (Table 1) were further tested for cytotoxicity against several tumor cell lines. Thus, spongianes **4**, **5**, **8**, **10**, **11**, **12**, the 2:1 mixture of **12/5**, and *ent*-isocopalanes **15** were evaluated in the tumor cell lines HeLa and HEp-2, the CHO cell line, and the bovine Bon-Fib primary cell culture; the CC_{100} values are shown in Table 2. Compound **5**, **12**, and the 2:1 mixture **12/5** were

Table 1. Cytotoxicity and Anti-HSV-2 Activity of Spongiane Diterpenes on Vero Cells[a] Determined the End-Point Titration Technique (EPTT)

spongiane diterpene	CC_{100} (μg/mL)[b]	viral reduction factor[c]	antiviral activity (μg/mL)[d]
1	40	N.A	N.A
2	50	$10^{0.5}$	25
3	56	10^{1}	28
4	28	$10^{0.5}$	14
5	9	$10^{0.5}$	4.5
6	58	$10^{0.5}$	29
7	37.5	10^{1}	18.7
8	28	$10^{0.5}$	14
9	120	$10^{0.5}$	60
10	28	N.A	N.A
11	28	$10^{0.5}$	15
12	8	N.A	N.A
12/5	7.5	10^{1}	1.8
13	>120	$10^{0.5}$	120
14	120	$10^{0.5}$	60
15	30	N.A	N.A
aciclovir	>600	104	6.0

[a] Vero, *Cercopithecus aethiops* african green monkey kidney ATCC CCL 81. [b] Minimal toxic dose that detached 100% of the cell monolayer. [c] Ratio of the virus titer in the absence over virus titer in the presence of the tested compound. [d] Maximal nontoxic dose that showed the highest viral reduction factor. N.A, no activity.

Table 2. In Vitro Activity of Spongiane Diterpenes against Cell Growth Expressed as CC_{100} (μg/mL)[a]

spongiane diterpene	cell lines[b]			
	HeLa	HEp-2	CHO	Bon-Fib
4	20	20	20	40
5	12	12	24	24
8	18	36	36	36
10	30	40	30	40
11	18	36	36	36
12	13	13	13	13
12/5	10	5	5	10
15	60	30	30	>120

[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; CHO, *Cricetulus griseus* Chinese hamster ovary cells ATCC CCL-61; Bon-Fib, primary culture of bivine ear subcutaneous fibroblasts.

the most toxic (CC_{100} of 12, 13, and 5 μg/mL on the tumor cell line Hep-2, respectively). According to the criteria established by the U.S. National Cancer Institute, the compounds with $CC_{50} < 4$ μg/mL are judged as active when they are incubated with the cells during 3 days.[14] To determine the CC_{50} values, all the compounds in Table 2, with the exception of the least active compound **10**, were examined for in vitro antitumor activity using the MTT method, but with an incubation period of 48 h. The results summarized in Table 3 again identified compounds **5**, **12**, and the 2:1 mixture **12/5** as the most cytotoxic spongiane analogues tested, with CC_{50} values of 4.4, 3.5, and 6.1 μg/mL on HEp-2 cells, respectively. Therefore, compound **12** appeared to be individually more selective for HEp-2 cells than in the mixture and at the same time was the most potent, which suggests that there was no synergistic effect with compound **5**.

On comparing the activities of **4** and **5**, it can be concluded that the presence of an α-acetoxy group in ring D clearly enhances the resultant cytotoxicity for the cell lines tested. Interestingly, the CC_{50} value on HeLa cells of **5** is quite significant since it was obtained in only 48 h on tumor cells of known resistance to chemotherapy. It is worth noting that this study is the first report on the

Table 3. Cytotoxic Activity of Spongiane Diterpenes Determined by the MTT Technique Expressed as CC_{50} (μg/mL)[a]

spongiane diterpene	cell lines[b]			
	HeLa	HEp-2	CHO	Bon-Fib
4	14.17 ± 1.0	9.56 ± 0.7	13.15 ± 0.7	18.98 ± 0.6
5	6.27 ± 0.3	4.43 ± 0.2	4.96 ± 0.6	18.3 ± 1.0
8	16.51 ± 0.6	10.05 ± 1.7	21.52 ± 1.8	15.86 ± 0.2
11	13.57 ± 1.2	10.42 ± 1.2	13.30 ± 0.6	17.92 ± 0.3
12	14.81 ± 1.1	3.46 ± 0.6	5.10 ± 0.5	18.86 ± 0.7
12/5	5.79 ± 1.2	6.07 ± 0.7	6.21 ± 0.4	21.96 ± 0.6
15	37.58 ± 4.5	21.83 ± 0.6	24.51 ± 1.1	>120
vincristine	0.05 ± 0.01	0.05 ± 0.01	1.15 ± 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; CHO, *Cricetulus griseus* Chinese hamster ovary cells ATCC CCL-61; Bon-Fib, primary culture of bivine ear subcutaneous fibroblasts.

biological activity of spongiane-type diterpenes possessing a formyl group at C-8.

The pentacyclic spongiane **12** also contains a lactone group in ring D, as does the lead compound **5**, but there is an additional hemiacetal ring system. Other derivatives of dendrillol-1 **12** possessing ester subtituents at C-7 such as aplyroseol-1 (7α-OCOPr)[5a] and aplyroseol-2 (7α-OCOMe)[3] have also shown a certain cytotoxicity against tumor cells, which suggests that this hemiacetal ring portion of the molecule is responsible for the biological activity. From our results, it can be concluded that the hemiacetal group attached to C-8 is essential to maintain the cytotoxic effect, since the esterification (compound **13**) or alkylation (compound **14**) of the corresponding hydroxyl group reduces the cytotoxicity. Again the importance of polar groups located at position C-8 in the spongiane skeleton seems to be proved, and these may be responsible for interaction with certain receptors on tumor cells. Finally, it is interesting to see that the *ent*-isocopalanes **15** were more selective against the tumor lines than against the Bon-Fib primary cell culture.[16]

These results encourage us to continue our research of this series by synthesizing additional spongiane-type derivatives with the aim of obtaining cytotoxic compounds that are more potent and selective toward tumor cells.

Experimental Section

Compounds. The diterpenoids tested were obtained following the procedures described by us.[7,8] The chemical structures of the compounds are shown in Figure 1. Stock solutions (7 mg/mL) of these compounds for testing in vitro were prepared in dimethyl sulfoxide (DMSO) and stored at 4 °C up to four weeks.

Cell Culture and Virus. The cell lines used were *Cricetulus griseus* Chinese hamster ovary cells (CHO cell line ATCC CCL-61), human cervix epitheloid carcinoma cells (HeLa cell line ATCC CCL-2), human larynx epidermoid carcinoma cells (HEp-2 cell line ATCC CCL23), and *Cercopithecus aethiops* African green monkey kidney cells (VERO cell line ATCC CCL-81). Bovine fibroblast primary culture cells of Bon criollo Colombian cattle (Bon-Fib) were obtained in our laboratory from bovine ear skin biopsies. Briefly the protocol used to obtain primary cell cultures from the biopsy was as follows. Each biopsy was washed three times with phosphate-buffered saline (PBS) containing 200 units/mL of penicillin, 200 μg/mL of streptomycin, and 0.5 μg/mL of amphotericin B. The skin was discarded, the cartilage and the subcutaneous tissue were minced finely, the pieces of tissue were placed in 25 cm^2 cell culture flasks with just enough growth medium, i.e., Eagle minimum essential medium (MEM) with 2 nM L-glutamine, 1% vitamins, 1% nonessential amino acids, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, and 10% of fetal bovine serum (FBS) to cover the pieces of tissue. When the fibroblasts had proliferated to 30 or 40% confluence, the pieces of tissue were discarded by gently shaking with PBS, and again the cells were fed with 50% of used medium and 50% of fresh medium. When 80% confluence was reached, the cells were trypsinized and cultured in 150 cm^2 flasks. Once the cells had covered about 80% of the surface, they were trypsinized, centrifuged, and cryopreserved.

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.

Herpes simplex virus type 2 (HSV-2) was obtained from the Center for Disease Control (Atlanta, GA). The virus stock was prepared from HSV-2-infected HEp-2 cell cultures. The infected cultures were subjected to three cycles of freezing–thawing and centrifuged at 2000 rpm for 10 min. The supernatant was collected, titrated in 10-fold dilutions by the plaque formation technique in microtiter plates, and stored at −170 °C in 1 mL aliquots. The virus titer was 4×10^5 PFU (plaque formation units) mL^{-1} or 16 MOI (multiplicity of infection).

Antiviral Assays. End-point titration technique (EPTT), the technique described by Vliectinck et al.[12] with a few modifications, was used. Briefly, confluent monolayer Vero cells were grown in 96-well flat-bottomed plates. Two-fold dilutions of the compounds in maintenance medium, identical to growth medium except for FBS which was 2%, were added 1 h before viral infection. The treated cells were infected with 10 infectious doses (1.6 MOI) of HSV-2 virus suspension and incubated again at 37 °C in humidified 5% CO_2 atmosphere for 48 h. Virus controls at each viral dilution (in untreated infected cells the MOI decreased from 1.6 to 1.6×10^{-5}), tissue culture controls (untreated uninfected cells), and compound controls (treated uninfected cells, to determine the cytotoxicity of the compound at each dilution) were included in each duplicate test. The antiviral activity is expressed as the reduction in virus titer at the maximal nontoxic dose of the tested compound or the highest concentration or lowest dilution that does not affect the monolayers in the conditions of the antiviral test procedure. Furthermore, all tests were compared with a positive control, Acyclovir (Quiviral), and tested simultaneously under identical conditions as reported previously (Betancur-Galvis et al., 1999). The reduction in virus titer was determined as the R_f of the virus titer, i.e., the ratio of the virus titer in the absence over virus titer in the presence of the compound of each the compound tested. Three assays were carried out in duplicate with at least five concentrations of each of the compound tested. The extracts with R_f values of 1×10^2 to 1×10^4 indicate a pronounced antiviral activity.

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 CHO cells and at 2×10^4 cells per well for Bon-Fib 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 a 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 from 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 EPTT assay. For the tetrazolium-dye (MTT)[13] cytotoxicity assays, the supernatants were removed from the wells, and 28 μL of a MTT (Sigma, 2 mg/mL) solution in PBS 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 (OD_{492}) 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.[14]

The results are obtained from triplicate assays with at least five compound concentrations. The percentage of cytotoxicity is calculated as $[(A - B)/A] \times 100$, where A and B are the OD_{492} of untreated and of treated cells, respectively.

Data Analysis. The 50% cytotoxic concentration (CC_{50}) for each compound was obtained from dose−effect curves (not shown).

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