Structure−Activity Relationship of in Vitro Antiviral and Cytotoxic Activity of Semisynthetic Analogues of Scopadulane Diterpenes

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Fourteen semisynthetic compounds derived from the natural scopadulane-type diterpenes thyrsiflorin A (**4**), B (**5**), and C (**6**), including several precursors, have been examined in vitro for their antiherpetic activity against Herpes simplex virus type II (HSV-2) and cytotoxicity against two human tumor cell lines. Four of these compounds showed moderate antiherpetic activity, but none of them exhibited a significant cytotoxicity against the cell lines used. Some structure−activity relationships have been identified for the antiviral activity in these scopadulane derivatives as well as important structural features for the cytotoxic activity.

Scopadulane diterpenes are members of a small group of plant metabolites recently discovered in the Scrophulariaceae family. Among those plants, *Scoparia dulcis*, has been known in Paraguayan traditional medicine for improving digestion and protecting the stomach, in Taiwan as a remedy for hypertension, and in India for toothaches and stomach disease.[1] In the course of the search for biologically active compounds from Paraguayan medicinal plants, Hayashi et al. reported the isolation and structure of two novel tetracyclic diterpenoids, scopadulcic acids A (SDA, **1**) and B (SDB, **2**),[2] from the 70% ethanol extract of the crude drug "Typychá-Kuratû" (whole plants of *S. dulcis*). The proposed structure of SDA was later confirmed by X-ray crystallography.[3] The same authors isolated a new SDB analogue, scopadulciol (SDC, **3**),[4] from *S. dulcis* collected in Taiwan which is believed to be identical to dulcinol that was found in *S. dulcis* from Bangladesh.[5] The plants of the genus *Calceolaria* (Scrophulariaceae family) available in various parts of Chile have also yielded several new diterpenes with the scopadulane skeleton. For example, thyrsiflorin A (TA, **4**), thyrsiflorin B (TB, **5**), and thyrsiflorin C (TC, **6**) were obtained from the chloroform extract of *Calceolaria thyrsiflora*.[6]

Since the scopadulcic acids were discovered in 1987, several of their pharmacological properties and possible therapeutic applications have been discovered. For example, these bioactive diterpenes inhibit in vitro gastric acid secretion and are considered potential antiulcer agents.[4] The in vitro antiviral activity of SDA, SDB, SDC, and other diterpenoids against Herpes simplex virus type I (HSV-1) has also been studied. SDB delays the appearance of herpetic lesions and prolongs the survival time of hamsters with HSV-1 corneal infection.[7] In addition, SDB has shown a potent cytotoxic activity against several tumor cell lines and against Ehrlich ascites cells inoculated in mice.[8]

The scopadulane diterpenes have structures similar to that of aphidicolin, a tetracyclic diterpenoid produced by the mold *Cephalosporium aphidicola*, which is a potent inhibitor of eukaryotic DNA polymerase α[9] and DNA polymerases induced by some viruses including HSV.[10] The mechanism through which the scopadulane diterpenes induce their cytotoxic and antiviral activities is unknown, but these activities may also be related to the inhibition of DNA polymerase(s). However, Hayashi and co-workers have shown that the antiviral effect of SDC could be attributed in part to the inhibition of viral protein synthesis in late steps of viral replication,[11] while SDB interferes at early events of virus growth.[7]

To date, the investigation of the structure−activity relationship of scopadulane diterpenes has mainly focused on inhibition of gastric H^+, K^+-adenosine triphosphatase (ATPase), which is the enzyme responsible for acid secretion in gastric mucous. Additionally, the studies performed on antiviral activity of SDA, SDB, and SDC have concluded that the carboxyl group at the C-18 in ring A together with the benzoyl group present at C-6 are necessary to maintain a relevant antiviral activity.[7,11]

In this paper we describe the in vitro antiviral and antitumor activity of various semisynthetic scopadulane-type diterpenoids and other related compounds. We establish new structure−activity relationships for these molecules, mainly in the C- and D-rings, and contribute to the characterization of their biological activity.

Results and Discussion

In this study we have evaluated the in vitro antiviral and cytotoxic activity of 14 semisynthetic compounds derived principally from the natural diterpenoids TA (**4**), TB (**5**), and TC (**6**) and their precursors. All of the following compounds were synthesized as single isomers from the chiral starting material (+)-podocarp-8(14)-en-13-one:[12] methyl thyrsiflorin A (**4a**, MTA), methyl thyrsiflorin B acetate (**5a**, MTBA), thyrsiflorin C (**6**, TC), thyrsiflorin C diacetate (**6a**), 13-scopadulanone (**7**), 13α-scopadulanol (**8**), 7β-hydroxy-13-scopadulanone (**9**), 7β-acetoxy-13β-scopadulanol (**10**), 8(14)-scopadulen-13-one (**11**), 8(14)-scopadulen-13,15-dione (**12**), 7α-hydroxy-8(14)-scopadulen-13-one (**13**), cyclopropane intermediate (**14**), 7α-hydroxy-8α-scopadulan-13-one (**15**), and the rearranged scopadulane-type diterpene ketone (**16**).

Initially, the cytotoxic activity and antiviral effect of all compounds were evaluated in a primary screening using the end-point titration technique (EPTT).[13] As shown in Table 1, compounds **7**, **8**, **11**, and **15** exhibited the highest antiviral activity (reduction factor of the viral titer of 10^2),

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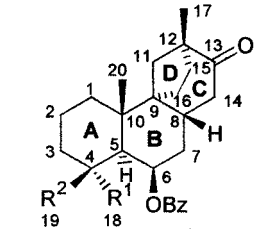
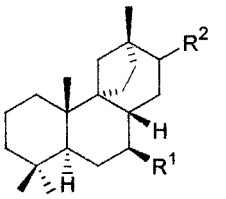
Table 1. Cytotoxicity and Anti-HSV-2 Activity of Scopadulane Diterpenes on Vero Cells[a] Determined by the End-Point Titration Technique (EPTT)

compound	CC_{100} (μg/mL)[b]	viral reduction factor[c]	antiviral activity (μg/mL)[d]
4a	30	$10^{0.5}$	15
5a	30	$10^{0.5}$	15
6a	50	$10^{0.5}$	10
6	30	N.A.	N.A.
7	28	10^{2}	14
8	32	10^{2}	7.5
9	48	$10^{0.5}$	10
10	50	10	25
11	40	10^{2}	20
12	60	10	30
13	28	10	14
14	40	10	20
15	60	10^{2}	30
16	35	N.A.	N.A.
acyclovir	>600	10^{4}	6.0

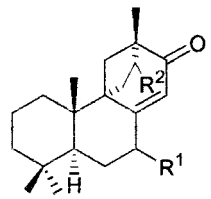
[a] VERO, *Cercopithecus aethiops* african green monkey kidney ATCC No. CCL 81. [b] The minimal toxic dose that detached 100% of the cell monolayer. [c] Ratio of the virus titer in the absence to virus titer in the presence of the tested compound. [d] The maximal nontoxic dose that showed the highest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



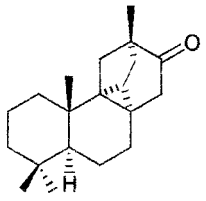
1 R^1= CH_2OH, R^2= CO_2H
2 R^1= CO_2H, R^2= CH_3
3 R^1= CH_2OH, R^2= CH_3



4 R^1= H, R^2= α-$OCOCH_2CO_2H$
4a R^1= H, R^2= α-$OCOCH_2CO_2Me$
5 R^1= OH, R^2= α-$OCOCH_2CO_2H$
5a R^1= OAc, R^2= α-$OCOCH_2CO_2Me$
6 R^1= OH, R^2= α-OH
6a R^1= OAc, R^2= α-OAc
7 R^1= H, R^2= =O
8 R^1= H, R^2= α-OH
9 R^1= OH, R^2= =O
10 R^1= OAc, R^2= β-OH



11 R^1= H, R^2= H
12 R^1= H, R^2= =O
13 R^1= α-OH, R^2= H



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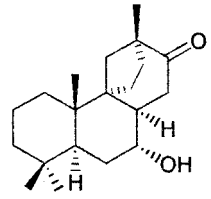

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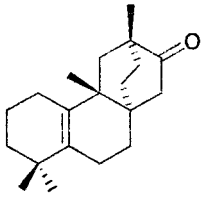

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indicating a moderate activity against HSV-2. In most of these compounds, the nontoxic concentration needed to obtain the largest reduction of the viral titer was approximately the same as the cytotoxic concentration needed to detach 100% of the cell monolayer (CC_{100}), revealing that their antiviral activity is principally due to their cytotoxicity. To clarify this aspect, it was necessary to calculate a selective index or therapeutic index for each compound with antiviral activity. The in vitro therapeutic index for each compound was calculated using the 50% cytotoxic concentration for cell growth (CC_{50}) and the 50% inhibitory concentration of the viral effect (IC_{50}), which was obtained by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method[14] (Table 2).

Table 2. Anti-HSV-2 Activity of Scopadulane Diterpenes on Vero Cells[a] Determined by the MTT Method

compound	CC_{50} (μg/mL)[b]	IC_{50} (μg/mL)[c]	therapeutic index[d]
7	16.7± 1.9	21.03± 0.9	0.81± 0.06
8	19.5± 1.2	16.5± 0.13	1.25± 0.29
11	23.2± 1.2	32.4± 4.1	0.72± 0.09
15	27.8± 5.9	42.1± 7.3	0.66± 0.19
acyclovir	12.2×10^4± 1.9	4.21± 0.9	

[a] VERO, *Cercopithecus aethiops* african green monkey kidney ATCC No. CCL 81. [b] 50% cytotoxic concentration. [c] 50% inhibitory concentration of the viral effect. [d] The therapeutic index is defined as CC_{50} over IC_{50}.

These therapeutic index values permitted us to establish accurately some structure−activity relationships as well as to identify important structural features responsible for the biological activity in the molecules. As shown in Table 2, compound **8** presented the highest therapeutic index (1.25). A polar substituent at C-13, as a hydroxyl group, is essential to preserve this activity since the presence of a carbonyl group at C-13 (compound **7**) showed a lower antiviral activity (therapeutic index 0.81), and there was complete loss of activity when the hydroxyl group was esterified (compound **4a**). Furthermore, the substitution at C-7 with a polar group led either to partial loss of activity (compound **10**) or even more to complete loss of activity, as observed with the natural product TC (**6**) (Table 1). The combination of the decreasing effects, that is, the substitution at C-7 with a polar group and the presence of a carbonyl or ester group at C-13, showed a complete reduction of the activity in compounds **5a**, **6a**, and **9**. The antiviral activity of compound **11** was very similar to that of compound **7**, which reveals that the double bond has little influence on activity. Interestingly, compound **15** showed anti-HSV-2 activity of the same order as compounds **7**, **8**, and **11** despite having the stereochemistry at C-8 inverted (8α-scopadulane structure) and a polar group at C-7. Finally, it is interesting to note that modification in the D-ring, as in compounds **12**, **14**, and **16**, also reduces the activity, suggesting that the D-ring spatial conformation is important.

Hayashi et al. reported the inhibitory activities of several derivatives of scopadulciol **3** against hog gastric H^+, K^+-ATPase and found that the potency of inhibition was higher for those compunds that contain an acetyl or oxime group at C-13. The importance of the substitution at C-13 to enhance biological activity is also confirmed by our results; however, to obtain better antiviral activities, the functionality should be a hydroxyl group instead of an acetyl group. To the best of our knowledge, this is the first study in which the structure−activity relationship in the C-ring of scopadulane diterpenes for their antiviral activity is evaluated. On the basis of our results and the results of others it can be predicted that the presence of a polar group at C-13 and no polar group at C-7, together with a carboxyl

Table 3. Cytotoxicity Data for **4a**−**6a**, **6**, **7**, and **13**[a]

scopadulane diterpene	cell lines[b]			
	HeLa	HEp-2	CHO	Bon-Fib
4a	28	28	28	28
5a	30	30	30	60
6a	40	40	40	60
6	45	45	45	60
7	28	24	24	24
13	20	20	20	10

[a] The minimal toxic dose that detached 100% of the cell monolayer (CC_{100} values (μg/mL) in 48 h). [b] HeLa, human cervix epitheloid carcinoma ATCC No. CCL 2; HEp-2, human larynx epidermoid carcinoma ATCC No. CCL 23; CHO, *Cricetulus griseus* ovary chinese hamster cells ATCC No. CCL 61; Fib-Bon, bovine ear skin primary culture.

group at C-18 and a benzoyl group at C-6, as found in other studies, may enhance the anti-HSV activity of these molecules.

For the most toxic compounds evaluated in the preliminary screening using the EPTT technique (Table 1), the CC_{100} values were obtained in the human tumor cell lines HeLa and HEp-2, in the CHO cell line, and in the Fib-Bon primary cell culture. As shown in Table 3, compound **13** was the most toxic, displaying on CHO cells and Fib-Bon culture CC_{100} values of 20 and 10 μg/mL, respectively. In general, all the compounds examined in this study did not show significant cytotoxic activity for the cell lines tested (the compounds with $CC_{50} > 4$ μg/mL are judged as inactive by the criteria established by the U.S. National Cancer Institute).[15] This weak in vitro antitumor activity was not important to be evaluated by the MTT quantitative technique, although in the light of our results (Table 3) it may be concluded that, in contrast with the antiviral activity, a polar C-7 substituent seems to be important for the cytotoxic activity, as in compound **13**. Concerning the cytotoxic behavior of this compound against either tumor cell lines or nontumor cell lines, on the basis of the results reported by Hayashi et al. and our results, it can be speculated that the substituents at C-18 and the benzoyl group at C-6 provide this molecule with certain cytotoxic selectivity against tumor cell lines.[8] These observations will, however, need further confirmation.

Experimental Section

Compounds. The diterpenoids tested were obtained following the procedure described by us.[12] Stock solutions (7 mg/mL) of these compounds for testing in vitro were prepared in dimethyl sulfoxide and stored at 4 °C.

Cell Culture and Virus. The lines cells used were *Cricetulus griseus* ovary chinese hamster 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). Fib-Bon primary culture cells were obtained in our laboratory from ear skin biopsies from pure BON cattle. Briefly, the protocol used to obtain primary cell cultures was as follows: the biopsy was washed three times with phosphate-buffered saline (PBS) containing 2% penicillin-streptomycin-amphotericine B, the skin was discarded, the cartilage and the subcutaneous tissue were minced finely, and the pieces of tissues were placed in 25 cm^2 cell culture flasks with just enough growth medium (Eagle minimum essential medium (MEM) with l-glutamine 2 nM, 1% vitamins, 1% nonessential amino acids, 1% penicillin-streptomycin-amphotericine B, and 10% fetal bovine serum (FBS)) to cover the pieces of tissue. When the fibroblasts proliferated to 30 or 40% confluence, the pieces of tissues 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 covered about 80% of the surface, they were trypsinized, centrifuged, and cryopreserved.

All cells were grown as a monolayer culture in MEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 20 mg/mL glutamine, 0.14% $NaHCO_3$, and MEM nonessential amino acid and vitamins solution. The cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

HSV-2 was obtained from Center for Diseases 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, and stored at −170 °C in 1 mL aliquots.

Antiviral Assays. End-Point Titration Technique (EPTT). The technique described by Vanden Berghe[13] with few modifications was used. Briefly, confluent monolayers of VERO cells were grown in 96-well flat-bottomed plates. Two-fold dilutions of the compounds in maintenance medium, supplemented with 2% serum and antibiotics, were added 1 h before the viral infection. Cells were infected with 0.1 mL of serial 10-fold dilutions of the appropriate virus suspension and incubated again at 37 °C in a humidified 5% CO_2 atmosphere for a period of 48 h. Controls consisted of infected cells with HSV-2 serial 10-fold dilutions in the absence of the compounds, treated noninfected, and untreated noninfected cells. The antiviral activity was expressed as the maximal nontoxic dose (MNTD) of the test compound needed to obtain the virus titer reduction. The virus titer reduction was expressed as the reduction factor R_f (virus titer in the absence of the compound/virus titer in the presence of it). R_f was 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.

Antiviral Colorimetric Assay. VERO cell monolayers were grown in 96-well microtiter plates. Dilutions of the compounds, prepared as described above in the EPTT assay, were added 1 h before the viral infection. Ten infectious doses of virus were added to each well and incubated at 37 °C in a humidified 5% CO_2 atmosphere for a period of 48 h. Controls consisted of untreated infected, treated noninfected, and untreated noninfected cells. Furthermore all tests were compared with a positive control (Acyclovir) and tested simultaneously under identical conditions as reported previously.[16] The cellular viability was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric technique.[14] Briefly, the supernatants were removed from the wells, and 28 μL of a MTT (Sigma) solution (2 mg/mL 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 on a multiwell spectrophotometer (Titertek Uniskan).

Cytotoxicity Assay. Cell monolayers were trypsinized, 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 Fib-Bon 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 by duplicates of at least five dilutions for each of the compounds. The results are expressed as the mean obtained from three different assays.

Data Analysis. The 50% cytotoxic concentration (CC_{50}) and the 50% inhibitor concentration of the viral effect (IC_{50}) for each compound were obtained from dose−effect curves (not shown). The CC_{50} and IC_{50} are the average of four assays with five concentrations within the inhibitory range of the compounds. The therapeutic index or selective index was defined as CC_{50}/IC_{50}.

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