

Cytotoxic Screening Activity of Secondary Lichen Metabolites

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SUMMARY. Five depsides and nine depsidones were studied for their cytotoxic activity. Both groups of specific phenolic lichen metabolites were tested in cell culture of lymphocytes to which tritiated thymidine was aggregated, using colchicine as standard. A considerable cytotoxic effect was observed for all the compounds, being pannarin, 1'-chloropannarin and sphaerophorin superior to colchicine. It is proposed that the molecular structures involved and the possibility of establishing hydrogen bonds by the substituents on the aromatic rings, might be responsible for the different biological activities observed.

RESUMEN. "Búsqueda de Actividad Citotóxica en Metabolitos Secundarios de Líquenes". Cinco dépsidos y nueve depsidonas fueron estudiados para determinar su actividad citotóxica. Ambos grupos de metabolitos fenólicos obtenidos de líquenes fueron probados en cultivos celulares de linfocitos midiendo su capacidad para incorporar timidina tritiada, utilizando colchicina como estándar. Se observó un considerable efecto citotóxico para la mayoría de los compuestos, en particular pannarina, 1'-cloropannarina y sfaeroforina, que presentaron un efecto citotóxico aún superior que el de colchicina. Se propone que la estructura molecular y la posibilidad de establecer puentes de hidrógeno por parte de los sustituyentes de los anillos aromáticos podrían ser los factores responsables de las diferentes actividades biológicas observadas.

INTRODUCTION

A lichen is a stable, self-supporting, mutualistic symbiont involving a fungus and a microalga and/or a cyanobacterium¹. They produce characteristic secondary metabolites which are unique with respect to those of higher plants². Depsides and depsidones are two major and representative groups derived from the polyketide pathway³.

Several lichens have been used in folklore medicine since ancient times⁴. Comprehensive and interesting papers on the relationship between lichens and man have indicated the frequent occurrence of antibiotic, antimycobacterial, antiviral, analgesic and antipyretic properties

⁵⁻⁷. However, report about the cytotoxic activities of these phenolic compounds are scarce^{8,9}. Previously, we informed about the activity of usnic acid¹⁰.

This paper presents the results of cytotoxic testing of nine common depsidones and five depsides found during the chemical research of several lichen species collected in Continental Chile and in the Antarctic territory.

MATERIAL AND METHODS

Plant material

The tested metabolites arise from diverse lichen species collected in different localities of continental and insular Chile.

KEYWORDS: Cytotoxic activity, Depsides, Depsidones, Lichen Secondary Metabolites, Structure-activity relationship.

PALABRAS CLAVE: Actividad citotóxica, Depsidonas, Dépsidos, Líquenes, Metabolitos secundarios, Relación estructura-actividad.

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Extraction and isolation

General experimental details have been reported previously^{11,12}. The pure compounds were identified by spectroscopic techniques (IR, ¹H and ¹³C NMR and MS), and by comparison with authentic samples.

Cell culture

The lymphocytes were isolated from the spleen of rats (100-200 g), in sterile conditions¹³. The erythrolysis was performed in NH₄Cl solution. The lymphocytes were cultured at 3 x 10⁵ cells in 1 ml of MEM (Minimum Essential Medium Eagle, M 4767, Sigma), supplemented with NaHCO₃ and Fetal Calf Serum (FCS) (Gibco) from 5 to 10%, as well as penicillin (100 units/ml) and streptomycin (100 µg/ml).

The cultures were incubated at 37 °C in an incubator gassed with 5% CO₂. After 24 h of incubation, they were changed to a fresh medium, supplemented with FCS and PHA (phytohaemagglutinin)¹³⁻¹⁵. The cultured cells were maintained at 37 °C during 48 h.

To assess the antiproliferative effects of the agents against lymphocytes, 1 ml culture was established at 3 x 10⁵ cell/ml in a drug-free medium or a medium containing the drugs to be tested.

After a 48 h exposure to quantitative effects of drugs on cell proliferation, a 4 h pulse time with 2 µCi/ml of [³H] thymidine (sp activity 2 Ci/min) (MEM) was added to each well, and cultures were maintained during this time in the incubator.

Harvesting culture

The cells of each well were transferred to a fibre-glass filter disc (Whatman FG/A) and the filter with saline solution was washed with 5% trichloroacetic acid (TCA) and finally with methanol. Afterwards, a needle was used to transfer the filter disc to a glass beta vial and 10 ml of liquid scintillation counter were added. The results were expressed as follows: dpm thymidine incorporates in the test culture/dpm thymidine incorporates in the control culture.

DNA/methyl green assay

DNA methyl green (5 mg) obtained from Sigma was suspended in 25 ml of 0.05 M Tris-HCl buffer, pH 7.5, which contained 7.5 mM Mg SO₄ and was stirred at 37 °C with a magnetic shaker for 24 h. The samples (20 l) of the compound tested at the concentrations of 10 µg/ml were dispensed and 200 l of DNA/methyl green solu-

tion were added to each well. The initial absorbance was measured at 630 nm, with a Shimadzu UV/visible spectrophotometer. Samples were incubated in the dark, at room temperature. After 24 h, the final absorbance of samples was determined as above described. Readings were corrected for initial absorbance and normalized as a percentage of the untreated DNA/methyl green absorbance value.

Calculation Methods

All the calculations reported here were carried out using molecular mechanics and semiempirical AM1¹⁶ quantum mechanics methods. Molecular mechanics (MM) calculations were performed using an empirical atom-atom potential method designed by the San Luis group^{17, 18}. For the molecular orbital semiempirical calculations the AM1 method implemented in the standard version of MOPAC 6.0¹⁹ was used. We have previously reported the use of these methods for biological systems, with excellent results²⁰⁻²².

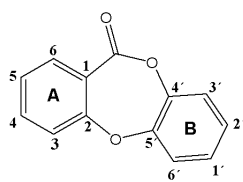
In a first step a systematic conformational search using the GASCOS method^{23,24} developed by the San Luis group was carried out. The compounds of interest were then modeled by the semiempirical AM1 method. After the structures were determined at semiempirical level of theory, molecular superimpositions between the most representative compounds of this series were carried out.

RESULTS AND DISCUSSION

Cytotoxic activity for depsidones (**1-9**), depsides (**10-13**) and the tridepside gyrophoric acid (**14**) was tested in cell culture of lymphocytes. The results are shown in Tables 1 and 2.

As main observations, it can be stated that many of the tested secondary metabolites displayed remarkable cytotoxic activity. Among them, compounds **1**, **2** and **11** have a cytotoxic effect stronger than colchicine, which was used as reference compound. In general depsidones displayed cytotoxic activities stronger than those obtained for depsides.

The analysis of the more active structures in depsidones (compounds **1-6**, Table 1) reveals that all of them possess and aldehyde group in C₃ and an adjacent hydroxyl group in C₄. In compound **6** these groups are located at C_{1'} and C_{2'}, respectively. By comparing these results with those obtained with depsidones displaying lower activity (compounds **7-9**, Table 1), it would appear that the presence of the above



Compound	dpm*	substituents							
		3	4	5	6	6'	1'	2'	3'
1	1.95	CHO	OH	Cl	Me	Me	H	OMe	Me
2	1.93	CHO	OH	Cl	Me	Me	Cl	OMe	Me
3	4.7	CHO	OH	H	Me	-CHOH-O-CO-	H		CH ₂ OH
4	6.91	CHO	OH	H	Me	COOH	H	OMe	Me
5	6.34	CHO	OH	H	Me	Me	COOH	OH	**
6	5.77	H	OMe	H	COC ₄ H ₉	C ₅ H ₁₁	COOH	OH	H
7	34.9	Me	OH	Cl	Me	Me	Cl	OMe	Me
8	11.6	CHO	OMe	H	Me	-CHOH-O-CO-	OH		Me
9	22.6	H	OH	H	Me	H	-CO-O-CH ₂ -		H
colchicine	3.5	-	-	-	-	-	-	-	-

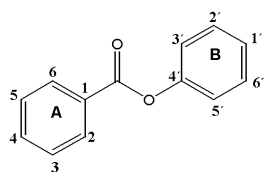
Table 1. General structural features of depsidones. The cytotoxic activity of compounds is expressed as dpm*. (**1**) pannarin, (**2**) 1'-cloro pannarin, (**3**) salazinic acid, (**4**) psoromic acid, (**5**) fumarprotocetaric acid, (**6**) lobaric acid, (**7**) vicanicin, (**8**) stictic acid and (**9**) variolaric acid. * dpm thymidine incorporated in the test culture/dpm thymidine incorporated in the control culture. ** CH₂-O-CO-CH=CHCOOH

groups seems to play a role to produce a significant cytotoxic effect. Thus, the strong hydrogen bond displayed between the aldehyde and hydroxyl groups could play a key role for the biological response. The lower cytotoxic activity displayed by compound **8** is in agreement with these observations. Note that the principal structural difference between compounds **3** and **8** is that the OH group in C₄ is methylated in compound **8** and therefore the hydrogen bond interactions cannot take place in this molecule.

It should be observed that compound **6** has

not the potentially reactive sites in ring A; however it still shows a remarkable activity. In this case, the presence of a COOH group in C₃ and a OH group in C₂ (ring B) could be mimetizing the chemical behavior of the aldehyde and OH groups (ring A) of the rest of the depsidones with cytotoxic effect.

According to the results obtained for the depsides compounds **10-13** (Table 2), we could attribute, at least in part, the cytotoxic activity to the presence of a COOH group in C₁ and a OH group in C₂ (ring B).

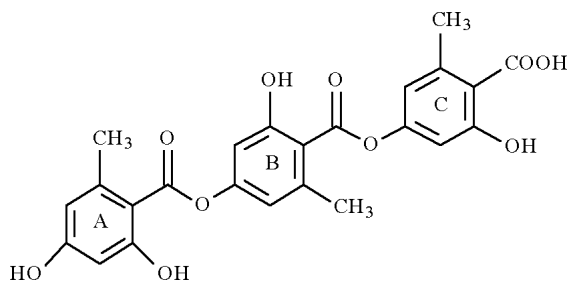


Compound	dpm*	substituents									
		2	3	4	5	6	1'	2'	3'	5'	6'
10	5.21	OH	CHO	OH	H	Me	COOMe	OH	Me	H	Me
11	2.27	OH	H	OMe	H	Me	COOH	OH	H	H	C ₇ H ₁₅
12	7.55	OH	H	OMe	H	C ₃ H ₇	COOH	OH	H	H	C ₃ H ₇
13	6.54	OMe	Me	OMe	H	Me	COOH	OH	Me	H	Me

Table 2. General structural features of depsides. Cytotoxic activity is expressed as dpm*. (**10**) atranorin, (**11**) sphaerophorin, (**12**) divaricatic acid and (**13**) difractaic acid. * dpm thymidine incorporated in the test culture/dpm thymidine incorporated in the control culture.

Particularly noteworthy was the activity obtained for compound **10**. This molecule has the COOH group in C₁ methylated and therefore the hydrogen bond with the OH of C₂ is not available. However, compound **10** is the only compound in the series (depsides) possessing an aldehyde group in C₃ and a OH group in C₄ (ring A) which might account for the cytotoxic activity obtained for this compound.

Gyrophoric acid (compound **14**) was the only tridepside tested in this report. This compound displayed a remarkable cytotoxic activity, however its biological response was about seven times lower than those obtained for compounds **1** and **2**. Compound **14** possesses a COOH group adjacent to an OH group in ring C. It is clear, however, that the structural feature in this compound is somewhat different to those of depsidones and depsides.



(14) gyrophoric acid (7.49 dpm)

To gain insight into the mode of action of these compounds in a second series of experiments, the ability of depsidones and depsides to disrupt the DNA/methyl green complex was evaluated by the DNA/methyl green assay. This assay is useful for the detection of agents that interact with DNA²⁵. Our results indicate that all the compounds tested were unable to displace

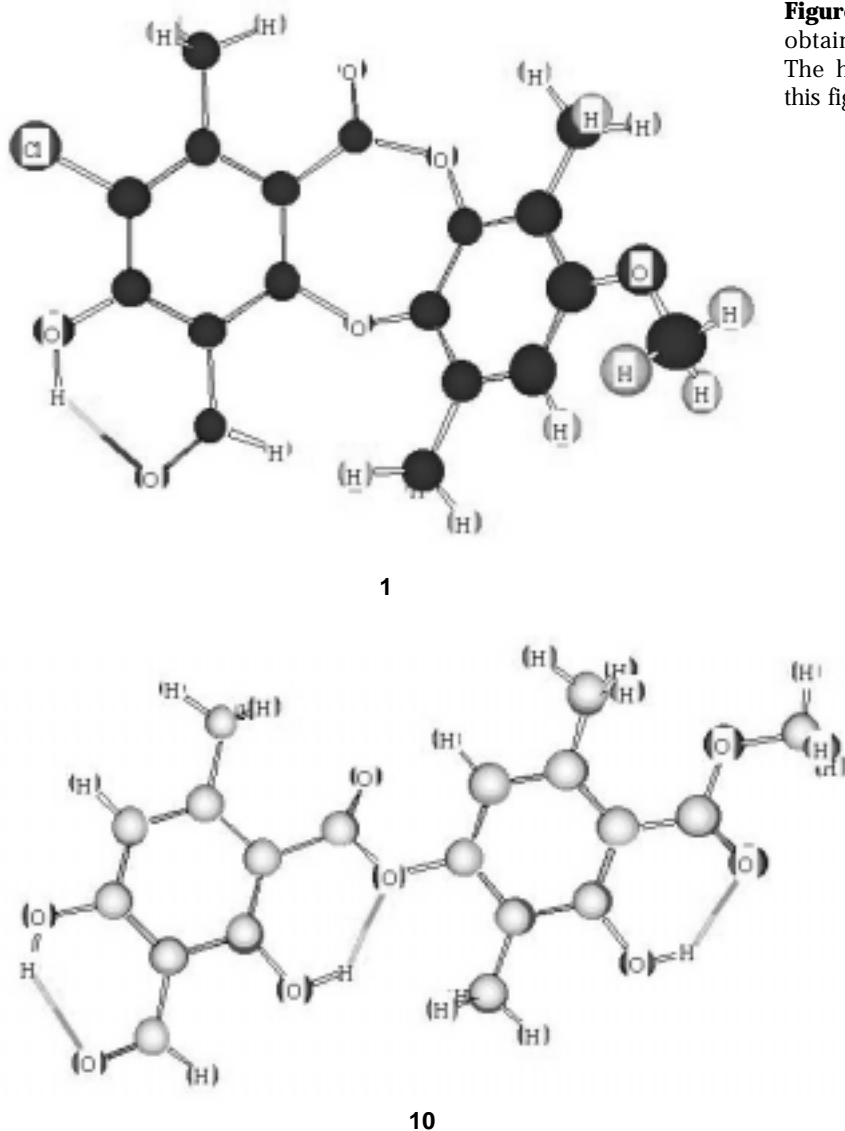


Figure 1. Low-energy conformations obtained for compound **1** and **10**. The hydrogen bonds are shown in this figure.

methyl green from the methyl green DNA complex. Thus it seems that the secondary metabolites reported here are not acting like intercalate agents at DNA level.

It is interesting to note that our results are in agreement with those previously reported for usnic acid¹⁰. It was reported that simple chemical changes which can modify the hydrogen properties of usnic acid, strongly influenced its cytotoxic properties.

For a better understanding of the above experimental results, we conducted a computer-assisted conformational and electronic study on the compounds shown in Tables 1 and 2. The purpose was to obtain more precise information as to how closely depsides resemble depsidones in terms of the spatial orientations of the essential components for biological response. However, the more detailed computational studies are beyond the scope of this report and, therefore, the results will be reported elsewhere.

Low-energy conformations of the compounds

were obtained from semiempirical AM1 calculations and compared. Figure 1 gives the low-energy conformations obtained for compound **1** and **10**. Compound **10** possesses three hydrogen bond stabilizing the conformation whereas compound **1** displays only one hydrogen bonding at the ring A. The molecular superimpositions of depsidone **1** and depside **10** is shown in Figure 2, in this superimpositions **1** has ring B almost coplanar with respect to ring A, whereas compound **10** has ring B raised about 60° with respect to the ring A. It is clear that **10** cannot place ring B in the same region space as the corresponding ring of **1**. However, both A rings, where the hydrogen bonds take place, fits very well. The similar spatial orderings and electronic distributions obtained for the potentially reactive sites of both depsidones and depsides suggests that these compounds interact at the biological receptor level in a chemically similar way. This is particularly apparent after examination of certain detailed stereochemical agreements.

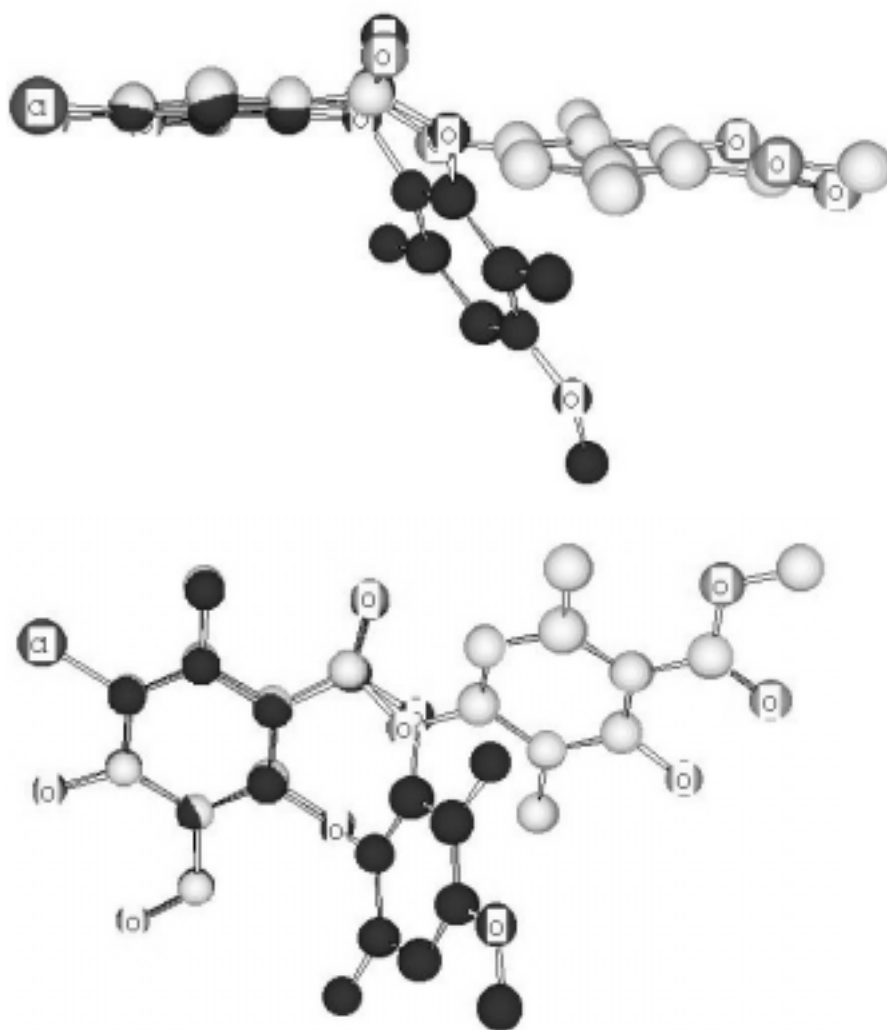


Figure 2. Superimposition of the lowest energy conformers of depsidone **1** (in black) and depside **10** (in light grey). The hydrogen atoms were deleted for simplification.

CONCLUSIONS

In summary, we report here a group of secondary metabolites (depsidones and depsides) acting as cytotoxic agents. Among them, compounds **1,2** and **11** and some of their structurally related congeners exhibited remarkable cytotoxic activity.

From an ethnopharmacological point of view, these results allowed the evaluation of cytotoxic effects of secondary metabolites obtained from lichens, which are commonly used in folklore medicine ⁴⁻⁷.

Furthermore, our results could provide useful information for the determination of the minimum structural requirements for the production of biological response by these compounds, indicating that the presence of COOH or CHO groups adjacent to an OH group appear to be indispensable.

On the other hand, although the molecular mechanism of the cytotoxic activity of the compounds reported here is unclear, the stereoelectronics complementarity observed among these molecules is noteworthy. Thus, on the basis of our results, it is reasonable to propose that a closely related chemical mechanism for the depsidones and depsides produces the biological response.

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