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SYNTHESIS AND CONFORMATIONAL ANALYSIS OF LEPTOCARPIN DERIVATIVES. INFLUENCE OF MODIFICATION OF THE OXIRANE RING ON LEPTOCARPIN'S CYTOTOXIC ACTIVITY.

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ABSTRACT

The reaction in acid conditions of Leptocarpin **1**, a compound with antitumor activity, formed two new isomeric products, 8 β -angeloyl-1 β ,3 β -dihydroxy-4,10-dimethyl,- $\Delta^{11(13)}$ methylen-4Z,9Z-dieneheliangol-6,12-olide **2** and 8 β -angeloyl-1 β ,3 β -dihydroxy-4-methyl- $\Delta^{11(13)}$, $\Delta^{11(14)}$ -dimethylen-4Z-eneheliangol-6,12-olide **3**, whose structures reported in this study were established by spectroscopy (¹H-NMR, ¹³C-NMR, MS and IR) and confirmed through ROESY experiments and theoretical studies by molecular mechanics. The *in vitro* cytotoxicity of these isomeric compounds was less active than leptocarpin, showing the importance of the oxirane ring in the biological activity. Cytotoxic activity was measured in six cancer cell lines.

Keywords: Conformational analysis; Heliangolide; Sesquiterpene lactones; Cytotoxicity.

INTRODUCTION

"Palo negro" is a native shrub of southern Chile, originally called Cüdu-mamëll (in Mapudungun) by the Mapuche people of Chile. Its scientific name is *Leptocarpus rivularis*¹ and belongs to the Compositae family, to the Heliantheae tribe, presently classified as Asteraceae². The secondary metabolites that characterize this family are acetylenics, sesquiterpene and sesquiterpene lactones. They also contain essential oils, flavonoids, and triterpenes but not tannins, iridoids nor amino acids nonprotein³. The extracts of plants containing antineoplastic activity have been profusely studied in the last decades⁴⁻¹⁰. A high percentage of the evaluated sesquiterpenes are potential inhibitors of cellular growth in numerous tumor models¹¹⁻¹⁴ and it has been suggested that the high cytotoxicity of some lactones may be due to their capacity to inhibit the synthesis and/or the transcription of DNA¹⁵. On the other hand, the protein synthesis is also affected¹⁶⁻¹⁷ and it has been demonstrated that the activity of sesquiterpene lactones- α,β -unsaturated is associated with the selective alkylation through a Michael-type reaction of biological nucleophiles such as L-cysteine or enzymes that control the cellular division. It is likely that these lactones inhibit the selective incorporation of amino acids¹⁸⁻²². The study of the effect of leptocarpin (Figure 1), a heliangolide type lactone isolated from *Leptocarpus rivularis*²³, on the macro-molecule synthesis of DNA, RNA and proteins in HeLa cell cultures²⁴, using Alonso's method²⁵ showed that leptocarpin and its corresponding acetylated derivative inhibit approximately 50% of the incorporation of 35S-Methionine without affecting the incorporation of (methyl-³H)-Thymidine and (5,6-³H)-Uridine at a concentration of 3.0×10^{-5} M. However, at concentrations lower than 6.5×10^{-6} M, it shows cytostatic effects. The reduction of exocyclic double bond in $\Delta^{11,13}$ causes inactivation of the biological activity of leptocarpin.²⁶

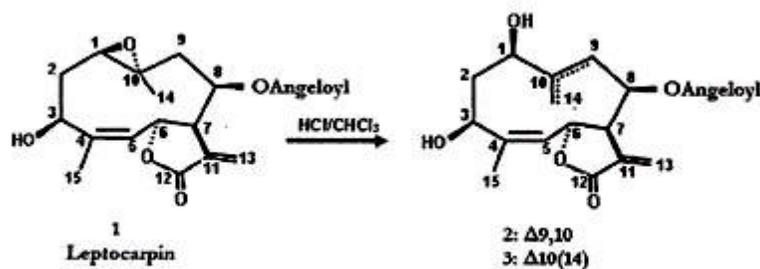


Figure 1. Acid treatment of leptocarpin with a saturated Chloroformic solution of HCl.

In this paper we report the synthesis of two sesquiterpene lactones leptocarpin derivatives that were designed to prove the importance of the oxirane ring in the biological activity of leptocarpin on cell uptake and cytotoxicity. These studies have provided fundamental data to assess whether leptocarpin could be a promising anticancer drug.

EXPERIMENTAL

Experimental procedures.

The ¹H-NMR spectra were taken in a spectrometer Brüker Avance DRX-400 at

400 MHz in CDCl_3 , and ^{13}C NMR (100 MHz). The IR spectra were obtained in a Perkin Elmer 200 spectrometer. The mass spectra (low resolution) were measured in a Vg-Micromass ZAB-2F spectrometer.

General Extraction Details.

Leptocarpin (**1**), is isolated and purified through usual laboratory techniques, extraction by ethyl acetate or ethanol and the obtained extract is purified using technical chromatographic methods²⁷ (silica-gel columns and solvents mixes of increasing polarity).

Hiisynthesis of compounds **2** and **3**.

The transformation of Leptocarpin was obtained through the acid treatment of the leptocarpin with saturated chloroformic solution with HCl, to mimick what would happen when the drug encounters the gastric acid pH (Figure 1). Five hundred milligrams of leptocarpin were treated with a saturated solution of HCl in CHCl_3 , shaking during 2 hours in an ice bath at 0°C . It was concentrated in a rotary evaporator, washed with a saturated solution of NaHCO_3 , and then with distilled water. The latter was extracted with chloroform and dried with anhydrous Na_2SO_4 . The products were separated and purified by preparative chromatography in silica-gel, eluted in a mixture of hexane/ethyl acetate (8:2), yielding 205 mg of recovered leptocarpin and 72 mg of a mixture of **2** and **3**. Attipts of purification by HPLC (Grad. Acetonitrile/ H_2O) failed. Analytical samples were obtained by several HPLC runs and preparative chromatography on Si-gel. The analysis of mixtures²⁸ by H-NMR revealed 6.9% of **3**.

8 β -angeloyl-1 β ,3 β -dihydroxy-4,10-dimethyl,- $\Delta^{11(13)}$ methylen-4Z,9Z-dieneheliangol-6,12-olide(2).

IR $\nu^{\text{Nujol}}\text{cm}^{-1}$: 3450-3400 GRATIS 3450-3400 (broad), 1772, 1736, 1659.
MS m/z (rel.int.): 362 M+ (6), 99 (10), 83 (98), 71 (12), 55 (100).
 $^1\text{H-NMR}$, δ : 1.80 (H-15,d, J=1.5Hz); 1.83 (H-14,d, J= 1.5Hz); 1.90 (H-5', d , J= 1.5Hz); 1.98 (H-4', d, J=7.0Hz); 2.19 (H-2, m); 3.12 (H-7, m); 4.53 (H-3, m); 4.80 (H-1, dd, J= 1.3, 3.5Hz); 5.30 (H-9, d, J= 9.0Hz); 5.42 (H-5, dd, J= 1.5, 3.5Hz); 5.80 (H-6, ddJ= 3.5, 10Hz); 5.95 (H-8, m, J= 3.97 and 9.24); 6.10 (H-13a, d, J= 1.¹Hz); 6.12 (H-3', dd, J= 1.5, 7.0Hz); 6.25 (H-13b, d, J= 1.¹Hz).
 $^{13}\text{C-NMR}$: δ : C-4', 16.89; C-14 20.71; C-15, 23.29; C-5', 26.86; C-2, 41.08; C-7, 50.25; C-8, 75.02; C-3, 76.68; C-1, 77.00; C-6, 77.32; C-9, 121.87; C-13, 122.81; C-3', 122.85; C-5, 127.13; C-11, 135.96; C-2', 139.82; C-4, 144.29; C-10, 146.29; C-1' 167.16; C-12, 169.80

8 β -angeloyl-1 β ,3 β -dihydroxy-4-methyl- $\Delta^{11(13)}$, $\Delta^{11(14)}$ -dimethylen-4Z-eneheliangol-6,12-olide (3)

IR $\nu^{\text{Nujol}}\text{cm}^{-1}$: 3445-3390 GRATIS 3445-3390 (broad), 1768, 1757, 1659.
MS m/z (rel.int.): 362 M+ (4), 182 (4) 99 (14), 83 (89), 71 (12), 55 (100).
 $^1\text{H-NMR}$, δ : 1.80 (H-15, d, J= 1.5Hz); 1.81 (H-5, d, J=1.5Hz); 1.96 (H-4', d,

J=7.0Hz); 2.08 (H-2, m); 2.55 (H-9, m); 2.90 (H-9', m); 3.20 (H-7, m); 4.10 (H-3, dd, J= 8.0, 0.3Hz); 4.50 (H-1, dd, J= 1.3, 3.5Hz); 5.12 (H-14, d, J= 1.3Hz); 5.16 (H-8, m); 5.32 (H-5', d, J= 1.5Hz); 5.50 (H-14', d, J= 1.3Hz); 5.75 (H-13a, d, J= 1.3Hz); 6.10 (H-3', m); 6.30 (H-6, m, J= 0.8, 3.0Hz); 6.33 (H-13b, d, J= 1.3Hz). ¹³C-NMR : δ: C-5', 18.92; C-15, 20.29; C-4', 22.89; C-9, 37.68; C-2, 41.44; C-7, 47.04; C-3, 76.68; C-1, 76.99; C-6, 77.20; C-8, 77.31; C-14, 117.90; C-13, 124.31; C-3', 126.31; C-5, 127.10; C-11, 137.41; C-2', 140.17; C-4, 141.71; C-10, 143.78; C-1', 166.68; C-12, 169.73.

General Cell Culture Details.

The studies on biological activity took place on cell cultures²⁹, such as NSO-2, CHO, P-815, HeLa, J 774.2, 4 FIO-67, SK-Hep-1. The cell lines were revived from liquid nitrogen, thawed and cultivated in RPMI 1640 culture medium (Chi.Sigma, USA) at 37°C in a humid atmosphere with 5% of CO₂. The culture was placed in 40 mL cell culture flasks. Once the cultures demonstrated a suitable growth, the cells were obtained by centrifugation (in the case of the adherent cultures, these were treated previously with Trypsin to detach the cells from the flasks). Following the centrifugation, the supernatant was discarded, the cells were suspended in 3 mL of RPMI 1640 medium with 10% of fetal bovine serum and antibiotics, counted, assessed for viability and incubated at a concentration of 2.5x10⁵ live cells/mL³⁰⁻³⁴.

Cell Uptake Studies.

The experiments of ³H-Thymidine, ³H-Uridine and ³H-Leucine uptake to DNA, RNA and proteins in the different cell lines with and without the studied compounds were carried out using 200 mL cell suspensions, plated out and incubated for 24 h at 37°C, in 5 % CO₂. Then, sesquiterpene lactones were added and incubated for further 72 h. Before culture termination, 0.2 μCi of ³H-Thymidine, ³H-Uridine and ³H-Leucine were added to each well respectively. After incubation, the cells were harvested onto a crystal filter paper using Multimash Harvested Dynatech Systi and processed for liquid scintillation counting (scintillation counter Packard instrument)³⁵. The liquid scintillation contained 0.5% of 2.5-difeniloxazol (PPO, Sigma Chemical Co) and 0.025% of 2,2'-fenil bis-(5-feniloxazol (POPOP, Sigma Chemical Co), in suitable solvent. The results were obtained in triplicate, expressed in counts per minute (cpm) and plotted using the program Graph Pad Prism 4.0.

Cytotoxicity Experiments.

Leptocarpin and its synthetic products were assayed for cytotoxic activity against SK-Hep-1 and HeLa cells. Cells (2.5 x 10⁵ cells/mL) were cultivated previously on plastics plates of 35 mm (Falcon) in the culture medium described. Cells were allowed to proliferate for 24 h and then treated with leptocarpin and its synthetic products **2+3**. The cultures were treated with trypsin at different times post treatment, suspended in 1mL of phosphate buffered saline (PBS) and the number of cells alive and dead was determined in aliquots of the cellular suspension. A mixture of 100 μL of cells suspension and 900 μL of trypan blue 0.1 % in PBS were incubated for five minutes. The total number of cells and the number of dead cells and stained with the vital dye were determined using a Neubauer

chamber (Assisant, Thoma model, Germany) under a microscope (Baush and Lomb, model 313364, Rochester, NY/USA).

Interestingly, for a same concentration of leptocarpin, a suspension of 2.25×10^5 cells (100 μL) showed 66% cellular death, while with a suspension of 1.25×10^5 cells (50 μL) an 83% of inhibition was achieved. The treatment with **2+3** compounds, showed a cellular death of 34% and 8% respectively under the same conditions. ([Table 5](#), [Figure 8](#)) respectively).

RESULTS AND DISCUSSION

Design and Synthesis of Derivatives.

The [Figure 1](#) shows the target products of the synthesis used in this study.

The opening of the oxirane ring was designed to prove the importance of this structural characteristic in the biological activity and hence to demonstrate if the human gastric acid pH is able to alter the leptocarpin structure. In order to determine the stereochemical course of the reaction, leptocarpin **1**, was added to chloroform saturated with HCl at 0°C for two hours. Analysis of the NMR spectrum of the crude product showed the presence of a significant number of impurities in addition to signals arising from **2** and **3**. Purification of the crude product by silica gel column chromatography allowed to recover non reacted material and to isolate a mixture of **2** and **3**. Purification of this mixture to obtain analytical samples of **2** and **3** by HPLC was difficult due to the close elution of these products and therefore, the isolation of pure analytical samples from the mixture required multiple cycles of HPLC and preparative TLC.

The structures were determined by H-RMN, ^{13}C -RMN and mass spectrometry, like the isomeric heliangolides ones, 8β -angeloyl- $1\beta,3\beta$ -dihydroxy-4,10-dimethyl-, $\Delta^{11(13)}$ methylen-4Z,9Z-dieneheliangol-6,12-olide (**2**) and 8β -angeloyl- $1\beta,3\beta$ -dihydroxy-4-methyl- $\Delta^{11(13)},\Delta^{11(14)}$ -dimethylen-4Z-eneheliangol-6,12-olide (**3**). The conformational analysis took place in CDCl_3 solution through ROESY experiments, being **2** of CC (Chair-Chair) type and **3** of TT type (Twist-Twist) which agrees with the theoretical studies of molecular mechanics using Hyperchi MM2 program. ([Figure 2](#)).

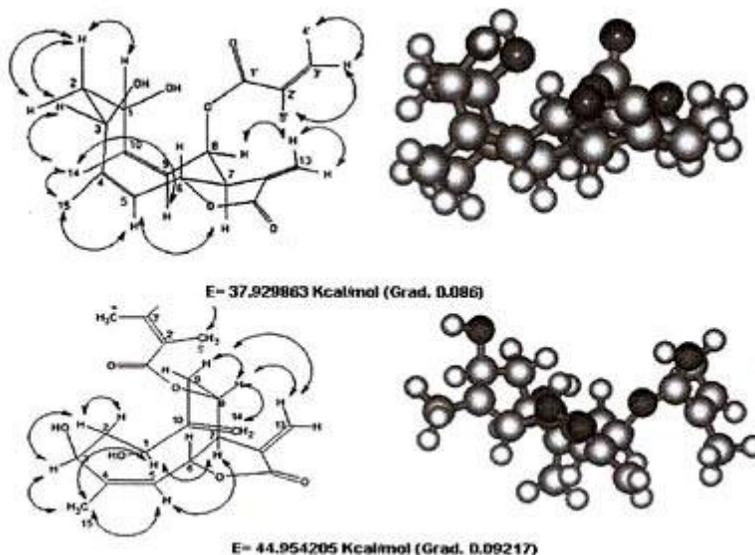


Figure 2. ROESY Experiments of 2 and 3.

From a theoretical point of view, leptocarpin under acid conditions can produce two types of cyclization products. First, an eudesmanolide type lactone by attack of the double bond $\Delta_{4,5}$ on C10 (6-Exo-Tet) producing the opening of the oxirane ring (Figure 3). The second product could be a guaianolide type lactone produced by the binding of carbons C5-C1 (5-Exo-Tet) as a consequence of the double bond $\Delta_{4,5}$ attack on C1³⁶. Nevertheless, this did not happen and a possible interpretation could be that interatomic distances C5-C10 (3.94 Å) and C5-C1 (3.26 Å) could be too far to allow the cyclization in each case.

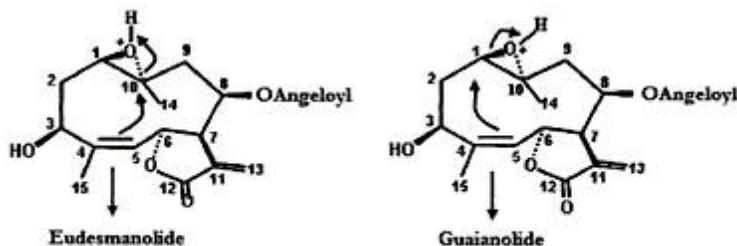


Figure 3. Structural types of sesquiterpene lactones expected from biogenetic theory by the acid treatment of Leptocarpin.

Contrary to the previous hypothesis, the acid treatment of leptocarpin (1) through the opening of the oxirane ring led to the formation of two heliangolide isomers, 8 β -angeloyl-1 β ,3 β -dihydroxy-4,10-dimethyl,- $\Delta^{11(13)}$ methylen-4Z,9Z-dieneheliangol-6,12-olide (2) and 8 β -angeloyl-1 β ,3 β -dihydroxy-4-methyl- $\Delta^{11(13)}$, $\Delta^{11(14)}$ -dimethylen-4Z-eneheliangol-6,12-olide (3) whose structures were confirmed by spectroscopy.

The conformational analysis of **2** and **3** was developed through ROESY experiments and molecular mechanics using the Hyperchi program. Thus, in ROESY experiments ([Figure 2](#)), they emphasize the interactions between protons H1-H2b, H2 β -H3, H5-H7 and H15, H8-H13, H9-H14, and protons of methyl, H14-H15, and between protons of the angeloyl group H3'-H4' and H3'-H15'. These interactions would be possible if **2** had a CC conformation as shown in [Figure 2](#).

On the other hand, the study of Molecular Mechanics through Hyperchi MM2 program ([Figure 2](#)) is coincident with the ROESY experiment. Of all the possible conformations, the CC shows the least conformational energy value that corresponds to 37.929863 Kcal/mol (Grad. 0.086).

With respect to the conformational study of **3** it is clearly observed in ROESY experiment the interactions between protons H1-H7, H1-H15, H2a-H3, H3-H15, H15-H5, H5-H7, H8-H9a, H8-H13b, H8-H14 and between H9a-H13b. Finally, the proton interactions of the angeloyl group H3'-H4' and H3'-H15' are also observed. All these interactions agree with a conformation TT as shown in [Figure 2](#).

The theoretical study by Molecular Mechanics of **3**, once again is consistent with the ROESY experiment since of all the possible conformations the most stable is TT conformation, with a conformational energy of 44.954205 Kcal/mol (Grad. 0.09217).

Solubility of compounds.

Leptocarpin (**1**) and its derivative compounds (**2**), (**3**) are soluble in dimethyl sulfoxide (DMSO) but insoluble in water. Aqueous DMSO is often used for the dilution of poorly water soluble compounds to *in vitro* cytotoxicity assays³⁷, up to a final concentration less than 5% of DMSO. Compounds **1**, **2** and **3** were soluble in 0.1% aqueous DMSO, which allowed us to perform *in vitro* biological studies on these compounds. The biological activity studies were carried out with the product mixture **2** and **3**.

Cell Uptake Studies.

The effect of leptocarpin and its acidic derivatives mixture on DNA, RNA and protein synthesis in five cancer cell lines, P-815, NSO-2, J 774.2, 4FIO-67, CHO, was examined by ³H-thymidine ([Table 1](#), [Figure 4](#)), ³H-uridine ([Table 2](#), [Figure 5](#)) and ³H-leucine uptake assays ([Table 3](#), [Figure 6](#)), respectively.

Table 1. (³H)-Thymidine uptake by different cell lines.

Cell lines	RPMI(cpm)	Lep (cpm)	2+3 Mixture (cpm)
P-815	80437 ± 3061	75414 ± 2734	75414 ± 2734
NSO-2	23234 ± 1778	23717 ± 2736	22250 ± 1713
J 774.2	41134 ± 1698	29843 ± 2876	22250 ±

			1713
4FIO -67	20733 ± 2481	15454 ± 2118	17416 ± 2478
CHO	29775 ± 664.7	29970 ± 1045	30303 ± 1531

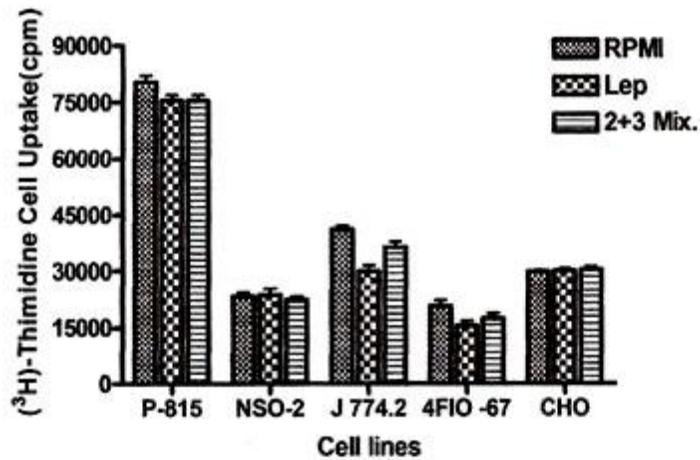


Figure 4. (3H)-Thymidine uptake by five cell lines in the presence of Leptocarpin or synthetic products 2+3 at 3.0×10^{-5} M.

Table 2. (3H)-Uridine uptake by different cell lines.

Cell lines	RPMI (cpm)	Lep (cpm)	2+3 Mixture (cpm)
P-815	65416 ± 1416	65007 ± 2298	61765 ± 1835
NSO-2	36055 ± 2683	35852 ± 1798	33996 ± 1554
J 774.2	28115 ± 2543	28029 ± 1229	27714 ± 1932
4FIO -67	27328 ± 2565	27501 ± 8808	27260 ± 2607
CHO	5369 ± 691.4	5729 ± 592.6	5632 ± 531.1

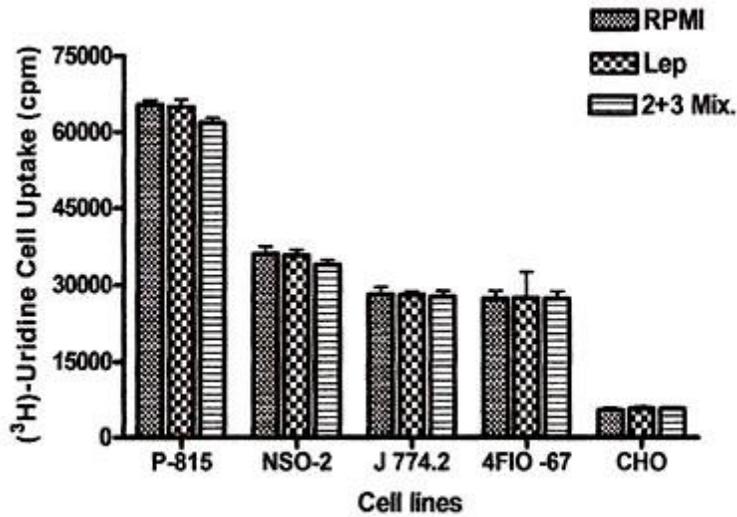


Figure 5. (³H)-Uridine uptake by five cell lines in the presence of Leptocarpin or synthetic products 2+3 at 3.0×10^{-5} M.

Table 3. (³H)-Leucine uptake by different cells lines.

Cell lines	RPMI (cpm)	Lep (cpm)	2+3 Mixture (cpm)
P-815	54343 ± 3922	26237 ± 1836	39477 ± 1300
NSO-2	29952 ± 3331	8650 ± 1643	23917 ± 3184
J 774.2	9964 ± 1651	4167 ± 294.3	7153 ± 860.6
4FIO -67	22022 ± 1933	11650 ± 1587	18343 ± 1027
CHO	6110 ± 871.2	2811 ± 215.9	3999 ± 174.9

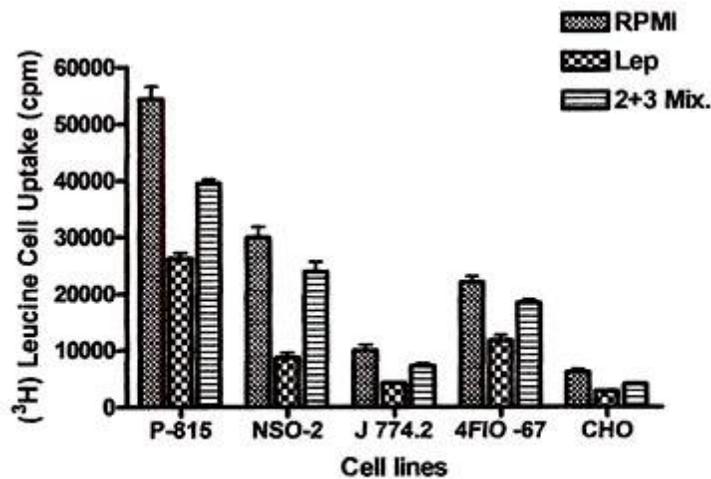


Figure 6. (³H)-Leucine uptake by five cell lines in the presence of Leptocarpin or synthetic products 2+3 at 3.0×10^{-5} M.

The results show that in all cancer cell lines investigated, the ³H-thymidine and ³H-uridine uptake were not affected by leptocarpin or its derivatives. Leptocarpin, however and clearly at a lesser extent its derivatives **2** and **3**, inhibited the ³H-leucine incorporation in every cancer cell lines examined. On the other hand, an increase in the concentration of leptocarpin or its derivatives had no effect on the thymidine and uridine uptakes.

Cytotoxicity Studies.

Leptocarpin (**1**) was assayed for cytotoxic activity against human adenocarcinoma cell lines (SK-Hep-1). One hundred µl of cells suspension were mixed with 900 µL of 0.1% trypan blue in PBS and incubated with leptocarpin for five minutes. The total cell count and the number of cells stained with trypan blue dye were determined using a Neubauer Chamber.

The effect of the Leptocarpin dose, in human liver adenocarcinoma cells SK-Hep-1, at different times of incubation was studied over a 48 hours period. Leptocarpin at 5.0×10^{-5} M induced a remarkable cytotoxic effect (12% of cell viability at 24 h); at 5.0×10^{-4} M it was cytotoxic showing cytolysis and cellular death a few hours following treatment (2.73% of cell viability at 24 h). At a concentration of 5.0×10^{-6} M it showed a cytostatic effect with IC50 value of 5 µM at 48 hours of treatment (Table 4 and Figure 7). The dose-response curves for the treatment of SK-Hep-1 cells with leptocarpin (**1**) for 24, 36 and 48 h are shown in Figure 7. It is clear that longer exposure times lead to increased cell death with very little reduction in viability observed in the cells treated just for 24 h with 0.1% DMSO and leptocarpin 5×10^{-6} M. The cells treated with leptocarpin 5×10^{-5} M and 5×10^{-4} M for 24 to 48 h showed similar levels of viability with very little cellular survival (12% and 2.73% of viability respectively at 24 h treatment). The cells treated for 48 h displayed 50% viability at a concentration of 5×10^{-6} M of leptocarpin (**1**).

Table 4. Cytotoxicity of Leptocarpin on human adenocarcinoma SK-Hep-1 cells (1.8×10^5 cells).

Treatment hours	0.1% Aqueous DMSO/Viability	Lep 5.0×10^{-6} M/Viability	Lep 5.0×10^{-5} M/Viability	Lep 5.0×10^{-4} M/Viability
24	89.9±1.88	86.2±2.19	12.0±1.06	2.73±0.21
36	91.4±2.61	56.1±1.62	7.56±0.58	1.50±0.26
48	90.1±2.84	46.9±1.81	2.56±0.50	0.93±0.15

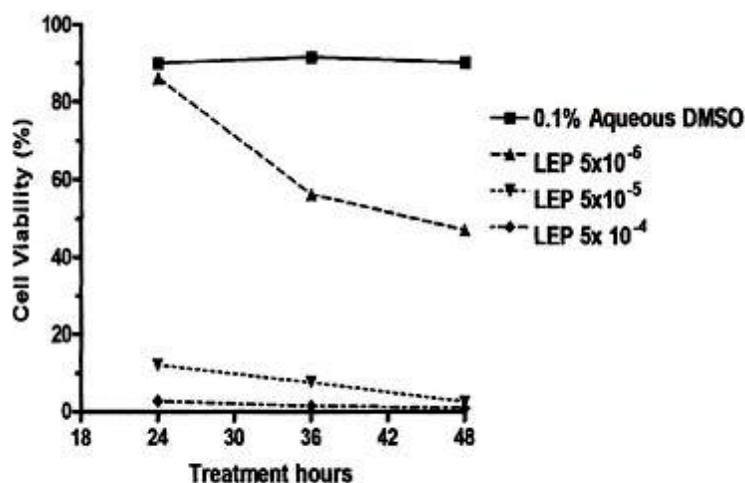


Figure 7. Kinetic of the effect of different concentrations of leptocarpin 1 on SK-Hep-1 cells viability.

The ability of leptocarpin (**1**) to inhibit the growth of human tumor cell lines was also assessed. HeLa cell suspensions of 2.25×10^5 and 1.25×10^5 cells exposed to a leptocarpin concentration of 3.0×10^{-5} M showed 34% and 16% cell viability, respectively, demonstrating the effectiveness of leptocarpin in the primary stages of a tumor. Using compounds **2** and **3** the results were 66% and 92% cell viability for the same cell suspensions. (Table 5, Figure 8).

Table 5. Cytotoxic effect of Leptocarpin 3.0×10^{-5} M on two different HeLa cell suspensions.

HeLa Cells	RPMI/Viability	Lep/Viability	2+3 Mixture/Viability
2.25×10^5	96.17 ± 2.12	33.83 ± 3.24	66.03 ± 2.25
1.25×10^5	96.03 ± 1.06	15.87 ± 2.80	92.2 ± 1.83

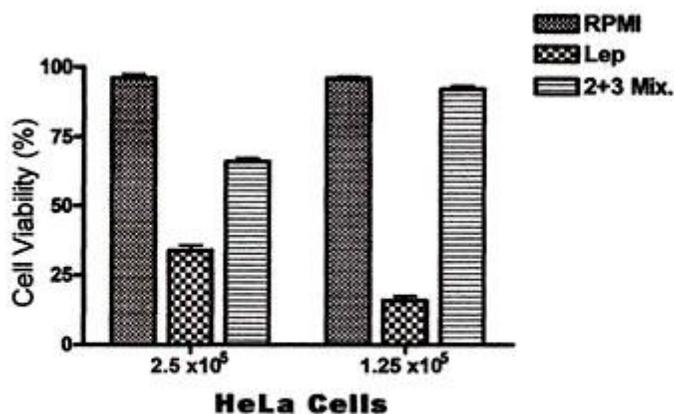


Figure 8. Effect of the treatment size dependence for two samples of HeLa cell suspensions with Leptocarpin and 2+3 compounds 3.0×10^{-5} M.

CONCLUSIONS

The transformation of leptocarpin is a relative straightforward process without any major technical problems for its synthesis, except for the separation and purification of the products.

DNA and RNA synthesis are not affected by Leptocarpin and the synthetic products therefore, its biological activity is not at this level. Leptocarpin produces a marked effect on the protein biosynthesis at a different extent in cancerous cellular lines and on the other hand, the synthetic products **2+3** have very low effect in comparison to leptocarpin thus demonstrating the importance of the oxirane ring for the biological activity.

The effectiveness of the results could determine that the leptocarpin is an antineoplastic agent of natural origin which can be used in different types of neoplasia.

The behaviour of Leptocarpin in an acid environment allows us to think that this molecule is sensitive to acid conditions and therefore as a possible future drug, it should not be administered orally, unless it is protected from gastric acid; this is why it is transformed into heliangolides structures with decreased biological activity.

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