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Cytotoxic cyclolignans related to podophyllotoxin

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Abstract

The cyclolignan family of natural products includes compounds with important antineoplastic and antiviral properties such as podophyllotoxin and two of their semisynthetic derivatives, etoposide and teniposide. The latter are included in a wide variety of cancer chemotherapy protocols. Due to these biological activities, cyclolignans have been the objective of numerous studies focused to prepare better and safer anticancer drugs. Several cyclolignans related to podophyllotoxin have been prepared and evaluated for their cytotoxic activities on four neoplastic cell lines (P-388, A-549, HT-29 and MEL-28); some of them have antiviral and immunosuppressive activities.

Keywords: Cyclolignans; Podophyllotoxin; Pyrazoline; Cytotoxic; Antineoplastic; Immunosuppressive

1. Introduction

Podophyllotoxin and many closely related lignans are known to have important antineoplastic and antiviral properties [1]. They have been the subject of numerous studies [2] focused on obtaining more potent and less toxic anticancer agents, which have resulted in the clinical introduction of etoposide and teniposide [3] and more recently, etopophos was also approved for clinical use [4] (Fig. 1).

The mechanism of the action of podophyllotoxin is related to its inhibition of microtubule assembly in the mitotic apparatus; it is a competitive inhibitor of colchicine binding to tubulin [5]. However, etoposide and teniposide were shown not to be inhibitors of microtubule assembly [6] which suggested that their antitumor properties were due to another mechanism of action and the essential nuclear enzyme topoisomerase II was identified as the target of these compounds [7]. Thus, it can be seen that changes in the configuration, size and chemical nature of substituents in the C ring of podophyllotoxin markedly affect the activity of the analogues. On the other hand, other podophyllotoxin derivatives, namely podophenazines, have also been reported to retain or even improve the cytotoxic activity, but these were weak inhibitors of topoisomerase II in vitro [8]. The data revealed that such analogues exhibit a different, as yet unknown, mechanism of action. More studies are needed to understand such mechanisms.

Over the past few years, we have been involved in isolation [9] and chemical transformations of podophyllotoxin and analogues and we have prepared a large number of cyclolignan derivatives by modifications of different positions in the cyclolignan skeleton [10–27], some of which displayed potent antiviral and cytotoxic activities [11,13,15,19,20,25]. Several of those derivatives modified in the C and D rings have been chosen for the analysis of antineoplastic effects of lignan analogues by modified CoMFA methods [28]. Also, several of those podophyllotoxin derivatives have a new heterocycle ring fused to the cyclolignan skeleton [16,22,24,29]. Actually, we are mainly preparing new compounds by modifications to the A and E cyclolignan-rings. They are being tested on cultures of different tumoral cell lines (P-388 murine leukemia, A-549 human lung carcinoma, HT-29 human colon carcinoma and MEL-28 human melanoma) and some of them have shown an interesting and selective cytotoxicity.

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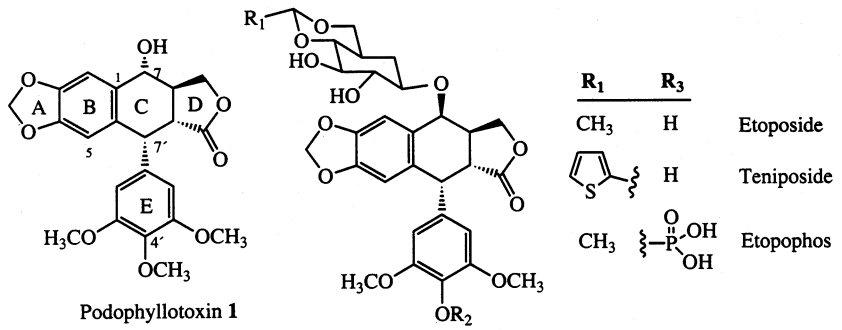


Fig. 1. Podophyllotoxin, etoposide, teniposide and etopophos.

2. Experimental

2.1. Chemistry

2.1.1. Pyrazolines, pyrazoles and isoxazolines [16,22,24]

The reaction between 7-ketocyclolignanolides and hydrazine derivatives is an easy method for the introduction of nitrogen substituents at that position. Rather than the expected hydrazones, but not surprisingly, the reactions led to a new family of compounds having a pyrazoline ring fused to the cyclolignan residue, while the lactone was opened to give a free carboxylic acid. Thus, podophyllotoxone **2**, prepared from **1** by oxidation with pyridinium dichromate (PDC), was converted in 65% yield (crystallized) into the pyrazoline-lignan **3** by reaction with phenylhydrazine in glacial acetic acid at room temperature [16]. Similarly, by reaction with the appropriate hydrazine derivative, other pyrazolines were prepared, which displayed electron attracting or withdrawing groups on the pyrazoline to prospect their respective influence on the antineoplastic activity [16].

To increase the types and number of compounds for testing we tried to transform the pyrazoline moiety into the corresponding pyrazole. Thus, PDC oxidation of **4** produced the aromatization of the heterocyclic ring leading to the alcohol **5**, isolated in 33% yield and the conjugated aldehyde **6** (31%) [16].

Similar to pyrazoline preparation, by reaction of podophyllotoxone with hydroxylamine in ethanol, isoxazopodophyllic acid **7** was obtained [22,24].

2.1.1.1. Preparation of pyrazoline derivatives. PDC (1.3 g, 3.45 mmol) was added to a solution of podophyllotoxin **1** (1 g, 2.4 mmol) in dry dichloromethane (25 ml) and stirred at room temperature for 4 h. The excess of PDC was removed by filtration followed by column chromatography of the residue on silica gel to give 750 mg (76%) of podophyllotoxone **2** and 200 mg (20%) of unreacted **1**. Phenylhydrazine (0.4 ml, 4.06 mmol) was added to a solution of **2** (1 g, 2.4 mmol) in 5 ml of glacial acetic acid and stirred at room temperature for 24 h. After addition of water, the unreacted ketone (90 mg) precipitated and was filtered off. The filtrate was treated with sat. aq. $NaHCO_3$ and extracted with ethyl acetate. After removing the solvent, 900 mg (74%) of phenylpyrazopodophyllic acid **3** was obtained [16]. In the way similar to that described for acid **3**, the following compounds were obtained using the corresponding starting materials: *m*-nitrophenylpyrazopodophyllic acid, *p*-bromophenylpyrazopodophyllic acid, methylpyrazopodophyllic acid, carbamoylpyrazopodophyllic acid and methyl acetylpyrazopodophyllate.

2.1.1.2. Preparation of pyrazole derivatives. A solution of alcohol **4** (115 mg, 0.24 mmol) in Cl_2CH_2 (5 ml) containing PDC (190 mg, 0.51 mmol) was stirred for 17 h. The crude product was purified by flash chromatography (CH_2Cl_2/EA 8:2 as eluant) to give the following compounds: phenyldehydropyrazopodophyllol **5** and phenyldidehydropyrazopodophyllal **6**.

2.1.1.3. Preparation of isoxazoline. Pyridine (0.2 ml) and hydroxylamine chlorhydrate (64 mg, 0.93 mmol) were added to a solution of **2** in ethanol (15 ml). The mixture was kept at 95–100°C for 72 h. Then the ethanol was evaporated off, diluted with EtOAc and washed with HCl (2 N) and brine to afford 310 mg of reaction product, from which 250 mg (81%) of **7** were separated after crystallization with CH_2Cl_2.

2.1.2. Synthesis of aldehydes with oxygen bridge [29]

We used as starting materials, the *trans*-lactone podophyllotoxin **1** and the *cis*-lactones picropodophyllin **10** and isopicropodophyllone **13**. These were transformed into the corresponding triols by reduction with LAH which is known to maintain the stereochemistry at the centers C_7, C_8 and $C_{8'}$. In the case of the reduction of **1**, the triol was accompanied by the dehydratation product neoanhydropodophyllol **8**. The formation of **8** can be explained by the presence of acid in the work up, because the corresponding triol can be transformed quantitatively into the alcohol **8** by keeping it under $CHCl_3$–HCl reflux. Reduction of **10** led

only to the triol which was transformed into neoanhydropicropodophyllol **11** when it was refluxed in chloroform solution acidified with a few drops of 2 N HCl. Reduction of **13** gave the triol as a mixture of epimers at C-7. They were also transformed into the dehydratation product **14** by refluxing them in $CHCl_3$–2 N HCl. In order to obtain the lignan derivatives with carbonyl group at C-9 and C-9′, Swern oxidation of the corresponding alcohols was performed and the aldehydes **9**, **12** and **15** were obtained from the neoanhydropodophyllols **8**, **11** and **14**.

*2.1.2.1. Neoanhydroisopicropodophyllal **15**.* About 180 mg (0.44 mmol) of isopicropodophyllone **13** in dry ether (15 ml) was slowly added to a suspension of $LiAlH_4$ (220 mg, 5.79 mmol) in dry ether. The reaction mixture was stirred at room temperature under argon for 3 h. Then wet EtOAc was added, filtered, dried and evaporated to afford 168 mg (92%) of the corresponding triol. A few drops of 2 N HCl were added to a solution of the triol (95 mg, 0.23 mmol) in $CHCl_3$ and heated under reflux for 1 h. After washing with water, drying over Na_2SO_4 and evaporation of the solvent, 90 mg (98%) of neoanhydroisopicropodophyllol **14** was obtained. *Swern oxidation*: to a precooled (-55°C) and stirred solution of oxalyl chloride (1.2 ml, 2 M) in dry CH_2Cl_2 (3 ml) was added dropwise 0.34 ml of DMSO in dry CH_2Cl_2 (3 ml). After 5 min at -55°C, a solution of 320 mg (0.8 mmol) of the alcohol **6** in 5 ml of dry CH_2Cl_2 was slowly added. The reaction mixture was kept at the same temperature for 30 min, then triethylamine (1.1 ml) was added. The mixture was warmed to 0°C over 1 h, quenched with water and extracted with CH_2Cl_2. The reaction product gave 252 mg (79%) of the aldehyde neoanhydroisopicropodophyllal **15** after flash chromatography (CH_2Cl_2/EtOAc 8:2 as eluant). In the same way as described for **15** and after column chromatography of the reaction product, the aldehydes **9** and **12** were obtained using the corresponding starting materials.

2.2. Antineoplastic assays [30]

Cells were seeded into 16 mm wells (multidishes NUNC 42001) at concentrations of 1×10^4 (P-388), 2×10^4 (A-549, HT-29 and MEL-28) cells/well, respectively, in 1 ml aliquots of MEM10FCS medium containing the compound to be evaluated at the concentrations tested. In each case, a set of control wells was incubated in the absence of samples and counted daily to ensure the exponential growth of cells. After three days at 37°C, under a 10% CO_2, 98% humid atmosphere, P-388 cells were observed through an inverted microscope and the degree of inhibition was determined by comparison with the controls, whereas A-549, HT-29 and MEL-28 were stained with crystal violet before examination.

3. Results and discussion

3.1. Structure–antineoplastic activity relationship

The great diversity of the cyclolignans, the huge number of assays carried out on them, and the different mechanisms of action observed in the different series make it difficult to clearly define the minimum structural requirements necessary for their biological activity. Additionally, the results available have been obtained by different authors, at different times and using different technologies, and on very diverse types of tumors or cultures of neoplastic cells. For all these reasons, greater systematization would be required to obtain definitive conclusions.

Recent developments on podophyllotoxin have afforded structure–activity relationships (SAR) which have assisted in the design and synthesis of new podophyllotoxin derivatives with potential antitumor activity. These SARs referred to as etoposide have been reviewed by Damayanthi and Lown [2b].

Our research team has isolated several cyclolignans from *Juniperus sabina* and has been and is still involved in the molecular manipulation of podophyllotoxin and related cyclolignans and nearly all of these derivatives have been evaluated for their antineoplastic activity. Below the main changes in the structure and their influence in the cytotoxicity are collected. To facilitate their study, they have been grouped on the basis of the ring of the cyclolignan skeleton that has been modified [27].

3.1.1. A-ring modifications

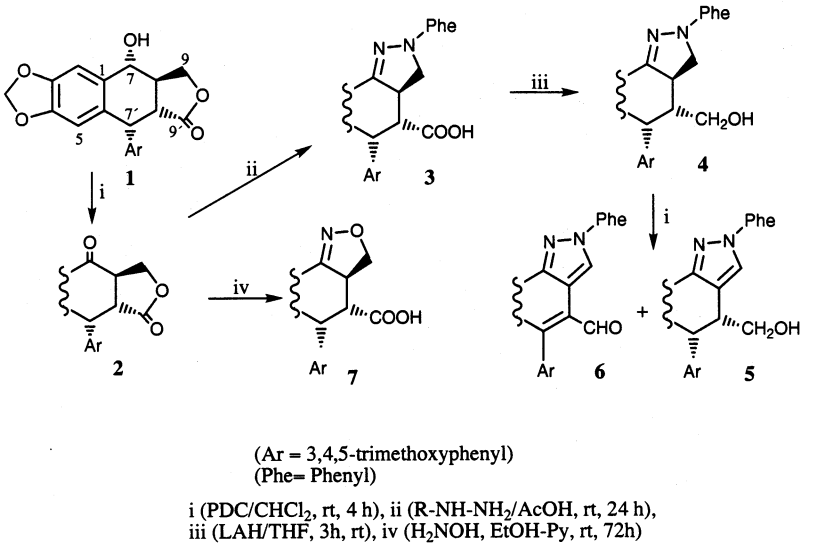
These include derivatives in which the methylenedioxy groups have been removed, the two phenol groups remaining free, which, in turn, have been transformed into other groups or oxygenated rings, with or without substituents [22,24]. Some derivatives without the methylenedioxy group, in particular the derivative of epipodophyllotoxin, are potent immunosuppressive agents [22,24].

3.1.2. B-ring modifications

Mention should also be made of some natural derivatives, such as peltatins, which are among the most potent and cytotoxic cyclolignans [9a,b,11,13,21].

3.1.3. C-ring modifications

Many and widely varying radicals have been introduced at position 7 in podophyllotoxin and epipodophyllotoxin. Reports have been made of compounds with oxygenated substituents in the form of ethers, esters and diverse nitrogen radicals [9a,b,11,13–15,17,21,22,24]. Other modifications to this part of the molecule include oxidations, as in the case of podophyllotoxone and some derived oximes. Elimination of the



Scheme 1. The synthesis of pyrazoline, pyrazole and isoxazoline derivatives of cyclolignans.

hydroxyl group at C-7 affords double bonds that may be found at any of the three possible positions [15,22,23,25].

3.1.4. D-ring modifications

Reports have been made of *cis*- and *trans*-lactone isomers, appearing either naturally or synthesized by transformations and interconversions, although from the point of view of activity the most interesting are the *trans*-lactone [9b–d,11,13,16–18,21–23]. Other modifications include the opening of the lactone ring, to give rise to compounds with different degrees of oxidation at positions C-9 and C-9′ [11,13–15,21,22]. In general, the derivatives lacking a lactone ring are less potent as antitumor agents.

3.1.5. C- and D-ring modifications

These include modifications involving the C and D rings and these have perhaps given the most promising results on activity obtained by our team. We should first mention some derivatives that, although lacking the D ring, display another cycle formed by the union of positions 7 and 9′, they are the group of the so-called *neoderivatives* [11–14,21,26,29]. Next, there are different compounds that have a double bond in the C ring, lack the lactone grouping and have positions C-9 and C-9′ with different degrees of oxidation [11,13,15,21,22,25,26]. Of special importance is the group of derivatives which are selective cytotoxic agents for human HT-29 colon cancer and have afforded an international patent [20], some of them are now in the *in vivo* study phase [15,25,26].

This section also includes a group of lignans that have heterocyclic rings fused to the cyclolignan skeleton. We have called them pyrazolignans [11,13,16,21,22] and isoxazolignans [19,22–24], and they were obtained by reacting podophyllotoxone with differently substituted hydrazines and hydroxylamines. Some of these derivatives show immunosuppressive activity, especially isoxazopodophyllic acid. Aromatization of the C-rings involves the loss of stereochemistry at carbons 8 and 8′; this is a functionalization that is found both in some natural and many semisynthetic compounds [9d,10,11,13,21].

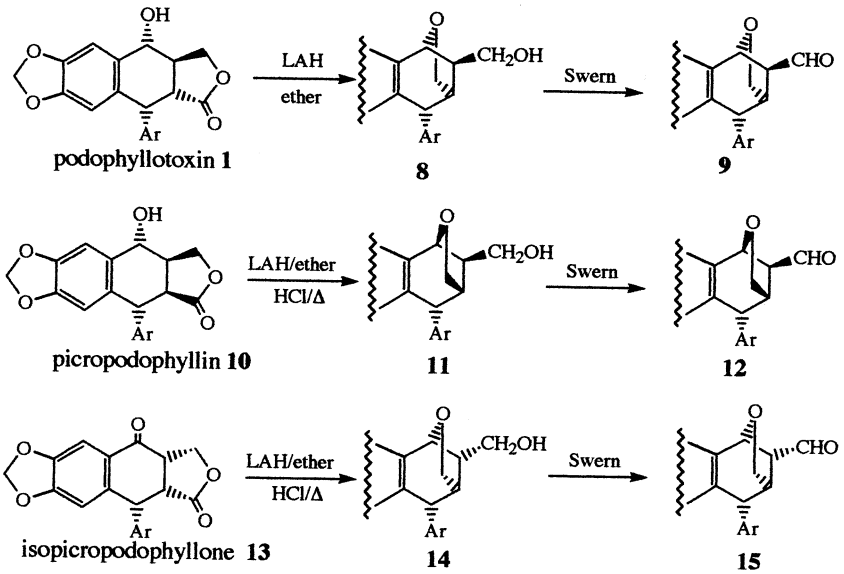
3.1.6. E-ring modifications

This part of the molecule has been related to lignan metabolism and its inactivation and, is perhaps the least modified part, although some transformations have been made, among which demethylations, oxidation of the *o*-quinone, the introduction of nitrogen radicals and increases in the coplanar rings can be mentioned [21,23].

Several of these derivatives can be obtained through simple chemical transformations from podophyllotoxin and analogues as shown in Schemes 1 and 2.

The prepared compounds were evaluated *in vitro* for establishing their cytotoxicity against cell cultures of P-388 murine leukemia, A-549 human lung carcinoma, HT-29 human colon carcinoma and some of them against MEL-28 human malignant melanoma [27] (Table 1).

For a better analysis of the influence of structural changes on the potency of these cyclolignans as antineoplastics, they have been grouped into three subclasses; namely *trans*-tetralinelactones, *cis*-tetralinelactones and non-lactonic cyclolignans. Two main observations could be noted. First, each individual substance showed similar potencies against the three or four neoplastic systems, thus inducing to assume a similar mechanism, probably related to its own citotoxicity, for all the compounds. Second, com-



Scheme 2. Synthesis of aldehydes with an oxygen bridge.

pounds contain a γ-lactone moiety showed, in general, IC_{50} values one to four orders lower than those for compounds which had not that moiety. Furthermore, within the lactonic compounds those displaying a *trans*-junction between the tetraline and lactone fragments were more potent than their *cis*-analogues [11,13,21].

Another important group of derivatives are those having heterocycles fused to the cyclolignan skeleton: pyrazoline and isoxazoline rings. All the isoxazoline derivatives were less potent than podophyllotoxin as antineoplastics, although some of them showed a very interesting immunomodulatory activity [19].

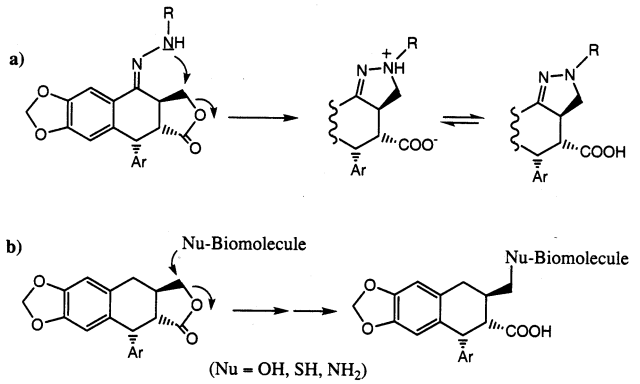
The pyrazoline and pyrazole derivatives tested show cytotoxic activity levels two and three orders of magnitude lower than those of podophyllotoxin, respectively, thus confirming that the presence of the lactone moiety is a prominent requirement for high cytotoxic activity to be achieved. Although some differences in potency can be observed within the family of *N*-phenylpyrazolines, subsequent analysis of the influence of the electronic character of the substituents on the phenyl group would have no significance [16].

The formation of a pyrazoline ring in the chemical condensation of phenylhydrazines with ketolactonic lignans can be interpreted mechanistically in terms of the nucleophilic attack of the second nitrogen atom of the hydrazone on the C-9 of the lignan. The attack should be facilitated by the strong tendency of the γ-lactone moiety to open, mainly due to the following reasons: (1) the ring strain caused by the *trans*-junction of the tetraline and lactone fragments; (2) the good leaving group nature of the carboxylate anion; and (3) the formation of a new, almost non-strained, pentagonal ring. It should be noted that the nitrogen atom participating in the nucleophilic substitution on C-9 is a poor nucleophile, due to its linking to the phenyl ring, which would tend to delocalize and disperse the negative charge constituted by the electron pair of the nitrogen atom.

Some molecular modeling studies have been carried out. These studies were based on the fact that these compounds are analogues of podophyllotoxin, a competitive inhibitor of colchicine binding to tubulin. Recent assessment of the structural basis for the inhibition of tubulin polymerization has concluded that the three-dimensional conformation of the A, B, C and D rings is important for interaction with tubulin [31]. For the studies, we have used the simplifying hypothesis that the site of action of all these compounds is largely

Table 1
Antineoplastic activity of cyclolignan derivatives (IC_{50} μM) — Cell lines: P-388 (lymphoid neoplasma from DBA/2 mouse); A-549 (human lung carcinoma); HT-29 (human colon carcinoma) and MEL-28 (human malignant melanoma)

Compound	P-388	A-549	HT-29	MEL-28
1	0.012	0.012	0.024	
2	1.8	1.8	1.8	
3	1.9	1.9	1.9	
4	4.1	5.2	5.2	
5	10	10	10	
6	21	42	42	
7	2.2	5.6	11	5.6
8	12.5	12.5	12.5	
9	0.3	0.6	0.6	0.6
10	6.0	6.0	6.0	6.0
11	6.3	12.5	12.5	
12	2.5	2.5	2.5	2.5
13	6.0	12.1	12.1	12.1
14	0.2	0.2	0.2	0.2
15	0.3	0.3	0.3	0.3



Scheme 3. Proposed mechanism for: (a) the formation of pyrazole derivatives from podophyllotoxone hydrazone; (b) the cytotoxic activity of cyclolignanolides.

occupied by podophyllotoxin, and that therefore the degree of overlap of each compound with podophyllotoxin could serve as a guide to expected activity. In this sense, the degree of overlap between podophyllotoxin and the selective cytotoxic cyclolignan-aldehydes were performed and according the results obtained [25], the unsaturated species is much better able to place the trimethoxyphenyl, the ester and the aldehyde in positions similar to those seen in podophyllotoxin than is the saturated system.

The group of naphthalene lactones and non-lactones does not seem to merit further analysis due to their proportionally much lower activity [11,13,21].

3.2. Mechanisms of action

Lignans inhibit the polymerization of tubulin and DNA topoisomerase II [1,2b,5–8]. This double inhibitory effect is not exerted in the same way by the lignans considered. Studies on SAR, have shown that podophyllotoxin-like compounds preferentially inhibit tubulin polymerization, which leads to the arrest of the cell cycle in the metaphase. However, etoposide-like compounds are potent irreversible inhibitors of DNA topoisomerase II and their action is based on the formation of a nucleic acid–drug–enzyme complex, which induces single- and double-stranded DNA breaks, as the initial step in a series of biochemical transformations that eventually lead to cell death.

On the basis of molecular modeling studies, Mac Donald et al. proposed a composite pharmacophore model [32]. According to this model, DNA topoisomerase II inhibitory activity, which is exhibited by various inhibitors such as daunorubicin, amsacrin and etoposide, would be due to the existence of three structurally distinct domains: a DNA intercalating moiety, the minor groove binding site, and the molecular region that can accommodate a number of structurally diverse substituents, which might also bind to the minor groove. One significant aspect of this composite pharmacophore model is that in order to be active, compounds do not have to interact with all of the three domains. Among these, only the minor groove binding domain and variable substituent accommodating region have been studied in depth [2b].

Eich et al. [33] carried out a structural proposal of the inhibition of DNA topoisomerase II by derivatives of epipodophyllotoxin, such as etoposide, consisting of the binding of the OH in position 4′ to the DNA through the phosphate unit of the nucleic acid and the formation of amides with topoisomerase II through the carbonyl group of the lignan, involving a covalent bond between the enzyme and the carbonyl group of the lignan with simultaneous breaking of the lactone. During our studies on cyclolignans [16] we have proposed that the cyclolignanolides of the podophyllotoxin group might work as alkylating agents through their C-9 methylene, rather than as acylating agents (Scheme 3).

Moreover, it is possible that lignan derivatives could act through a different mechanism from those described previously. Cyclolignan derivatives have been found in which the number of coplanar rings of the tetracyclic system is increased and yet they do not affect either tubulin polymerization or DNA topoisomerase II [8], indicating that further studies are required to elucidate this mechanism of action.

Another interesting fact to note in our cyclolignans is the general parallelism observed between antineoplastic and antiviral results. In spite of the differences observed for each system assayed, this could be interpreted in terms of the existence of a similar mechanism of these actions of cyclolignans against both cells and viruses.

3.3. Antiviral and immunosuppressive activities

The antiviral activity was performed against herpes simplex type 1 (HSV-1) and vesicular stomatitis (VSV) viruses infecting monkey kidney fibroblasts (CV-1) and hamster kidney fibroblasts (BHK), respectively [11,13,21]. As in the case of antineoplastic activities, we observed that *trans*-tetralinelactones are the most potent antiviral agents among the compounds and systems tested but, in this case, the difference in potency may be even greater.

The results obtained for both systems revealed a noteworthy degree of parallelism with the results on antineoplastic activity, such that the antiviral effect of these compounds is considered to be based on the actual cytotoxicity of the compounds themselves. None of the cyclolignans assayed showed any great activity against HIV-1

Several of our cyclolignans have been subjected to *in vitro* and *in vivo* evaluation of their immunomodulatory activity [22,24]. Of the structural modifications performed on the molecule of podophyllotoxin, demethyle-

nation of the dioxol grouping and opening of the γ-lactone ring, with simultaneous formation of a fused isoxazol heterocycle seem to be adequate transformations for retaining, or even increasing, the immunosuppressive activity of cyclolignan.

3.4. Conformational study

Antineoplastic and antiviral activities are greatly selective to certain classes of lignans, the *trans* and *cis*-tetralinelactones being much more active than those of naphtalene and non-lactonic cyclolignan classes. The differences in activity for both types of tetralinelactones could be explained in terms of their differences in conformation, induced by the change of configuration at C-8′. *Trans*-lactones are rigid, almost planar in the tetracyclic moiety, and display the aryl substituent at C-7′ in a free rotating pseudoaxial disposition, whereas *cis*-lactones are more flexible, with conformations ranging, depending on the nature of their substituents, between various folded dispositions of the fused four rings system with the 7α-aryl group axial and an extended, more planar disposition, with the 7α-aryl group being practically equatorial [11,13]

One other possibility for explaining the difference in potency for *cis* and *trans*-tetraline- lactones lies in the relative stability of both types of lactones. *Trans* compounds are more strained than the *cis* ones and hence they could be more reactive, thus showing a greater capacity to make covalent bonds with the target biomolecule in the biological system.

4. Conclusions

It has been possible to deduce that the biochemical mechanism of the anchoring of cyclolignans to DNA or topoisomerase II would not involve the nucleophilic attack of part of the biomolecule on the lactonic carbonyl at position 9′, but rather on the lactonic methylene at position 9, thus leading to breakage of the strongly strained γ-lactone and hence irreversible blockade of the biological substrate. Furthermore, interpretation of the results from the assays that have been carried out to date has allowed us to deduce the structural fragments that are most important for antineoplastic activity and the pathways permitting decreases in cellular toxicity and increases in selectivity have been established. This will lead to new proposals regarding possible improvements to the therapeutic indices of these types of compounds.

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