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IN VIVO IMMUNOSUPPRESSIVE ACTIVITY OF SOME CYCLOLIGNANS

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Abstract: Several podophyllotoxin-related cyclolignans, either lacking the lactone ring or the methylenedioxy grouping, have been prepared and evaluated for their immunosuppressive (IMS) activity in the mouse allogeneic MLR *in vitro* test and in the *in vivo* techniques Graft *vs* Host Reaction (GVHR) and Skin Grafting (SG). The results obtained show that three cyclolignans fairly prevent splenomegaly in comparison with control animals and also promoted tolerance to grafting, being the first time that the *in vivo* IMS activity of cyclolignas is reported.

Graft rejection remains to be the main obstacle of successful transplantation. Since cyclosporin A (CsA)[1] was introduced, the success in organ transplantations has increased remarkably. Recently, FK-506[2] was found to be 10-100 fold more potent than CsA. Other agents currently used as immunosuppressants include rapamycin, steroids and the inhibitors of the purine and pyrimidine biosynthesis.[3] Other simpler compounds with potent immunosuppressive activity have been isolated from culture broths of *Isaria sindiarii* and *Mycelia sterilia*.[4] All these agents, apart from an important proportion of failures, show a number of undesired side effects including nephrotoxicity,[5] hypertension,[6] neurological disorders,[7] gingival overgrowth,[8] hirsutism,[9] gastrointestinal disturbances,[10] etc. As a consequence, new compounds are undergoing extensive clinical trials to establish their efficacy and safety in the protection of transplanted patients[11] and the search for the discovery of new lead compounds continue to be of great interest.

In a previous paper we reported the results of a preliminary evaluation which showed the potential interest of cyclolignans as immunosuppressive (IMS) agents.[12] Following this research and assuming that substantial improvements of the therapeutical index for these compounds, would be attained better through decreasing their cytotoxicity rather than through the increase of their IMS potency, we looked at the structural facts being among the most relevant for their cytotoxic activity,[13] as the target structural points to be modified. We expected that elimination or substitution of the γ-lactone or dioxol rings would produce that improvement. Following this strategy a number of cyclolignans were selected for testing in the *in vitro* MLR method and on the basis of previous[12] and the new results described in this paper, the most promising compounds were then submitted to two types of *in vivo* evaluation; namely the Graft *versus* Host Reaction (GVHR) and the Skin Grafting (SG) methods. The results of such evaluations are shown and discussed in this paper, which represents the first report of *in vivo* evaluation of the immunosuppressive activity of cyclolignan derivatives. From the analysis of such results, it can be stated that cyclolignans have a great potentiality for being considered as promising lead compounds, for the development of clinically useful immunosuppressants, which could prevent organ rejection in transplants.

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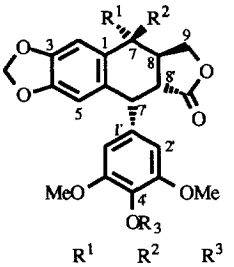
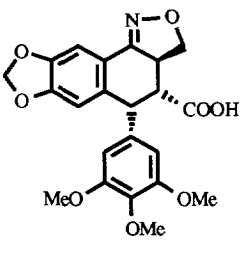
Chemistry

Some of the compounds tested and those used as starting materials, as it is the case of deoxypodophyllotoxin (**1**), podophyllotoxin (**2**) and 4'-demethylpodophyllotoxin (**3**), were isolated from *Podophyllum* resin by chromatographic procedures.

Deoxypodophyllotoxin (**1**), was transformed into **4**, which has a free phenolic hydroxyl group at C-4',[14] by demethylation with 33 % HBr-AcOH.[12] **5** was also obtained from **1** by aromatization with DDQ.[15] **2** was transformed into **6**[16] and **7**[12] by described procedures.





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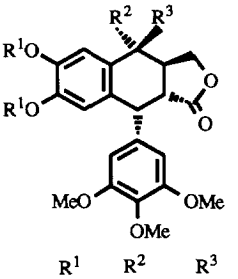

	R^1	R^2	R^3	
1	H	H	Me	
2	OH	H	Me	
3	OH	H	H	
4	H	H	H	
5	H	H	Me	$\Delta^{7,8'}$
6	H	H	Me	$\Delta^{8(8')}$
12	H	OH	Me	

	R^1	R^2	R^3	
8	H	H	H	
8a	Ac	H	H	
9	H	OH	H	
9a	Ac	OAc	H	
10	H	H	OH	
10a	Ac	H	OAc	
11	H	H	H	$\Delta^{8(8')}$
11a	Ac	H	H	$\Delta^{8(8')}$

The preparation for compounds **8-11**, which belong to the group lacking the methylene group of the dioxol ring, is summarized in table 1.[15,17,18] Boron trichloride was used for attaining the cleavage of the methylenedioxy group selectively, in presence of the aromatic methoxyl groups of the trimethoxyphenyl moiety. The cleavage of the methoxy groups usually requires either higher temperature or longer reaction time.[19] The best yields in demethylenated compounds were obtained when the reaction mixture was kept one hour at low temperature and one hour under reflux in presence of calcium carbonate. After longer reaction periods, 3,4-demethylenepodophyllotoxin (**9**) was accompanied by two other compounds, its 7-epimer **10** and the dehydration product **11**.

Compound **11** was also the major product of the acid-catalyzed cleavage of the methylenedioxy ring of **2** according to Schreier's method.[20]

Table 1. Preparation of compounds 8 to 11

Substrate	Method[a]	Temperature	Time (i+ii)	Products
1	A	-78 °C	75 min + 75 min	**8** (83 %)
2	A	-65 to -70 °C	1 h + 1 h	**9** (80 %)
2	A	-78 °C	2.5 h + 4h	**9** (11 %) + **10** (16 %) + **11** (14 %)
2	B	150 °C	3 h	**11** (43 %)

[a] Method A: i. BCl_3-CH_2Cl_2, low temperature; ii. H_2O, acetone, $CaCO_3$, reflux. Method B: H_3PO_4, Phenol, AcOH

Biological Results and Discussion

The mouse allogeneic MLR method has been recently used by us to perform the *in vitro* preliminary evaluation of IMS activity of cyclolignans.[12] Now, it is applied to compounds **6, 8-11** in the same way. The results obtained (table 2) really confirmed our hypothesis that the disappearance of the methylenedioxy moiety of podophyllotoxin-related cyclolignans would improve their therapeutical IMS index, as it can be seen for those demethylenated derivatives of podophyllotoxin **9** and epipodophyllotoxin **10**. The IMS data for podophyllotoxin and its 7-epimer epipodophyllotoxin has also been included in table 2 for comparison purposes.

Table 2. *In vitro* Immunosuppressive activity and Cytotoxicity Data for compounds 6-11a

Compound	MLR[a]	LcV[b]	nCIMI[c]
2	<0.128	280	>2187
6	<0.00855	>28.5	>3333
7	<0.009	>30.0	>3333
8	0.585	>25.0	>43
9	0.00563	>27.5	>4885
9a	0.0607	>26.0	>428
10	0.0000015	>35.0	>23333333
10a	0.427	>28.5	>67
11a	0.366	>28.5	>78
12	0.229	>65.0	>284
CsA	0.0518	2.75	53

[a] IMS activity. IC_{50} (μg/mL). [b] cytotoxic activity (μg/mL). [c] Non-cytotoxic IMS activity (ratio LcV/MLR) Eight different dilutions were tested for every compound (conc. ranged from 0.5 to 0.0015 mg/mL approximately)

It can be appreciated that cellular viability values remain within the same order of magnitude, while the MLR values show a much greater interval, thus producing great differences in the nCIMI index. It can be also observed that acetylation reduces IMS activity by one order of magnitude in the case of **9** and **9a** and much more in the case of **10** and **10a**. The extraordinarily high MLR values for **10** in comparison with those observed for other structurally close compounds, is really quite surprising.

As a consequence of this and previous[12] results, the oxazoline-fused derivative **7**, lacking the lactone ring but possessing the methylenedioxy function and compounds **9** and **10**, having intact the lactone ring by lacking the methylenedioxy group, were selected for *in vivo* evaluation. Firstly, the selected compounds were assayed in the GVHR test, which evaluates a cell-mediated type response and represents the direct *in vivo* correlate of the *in vitro* MLR assay and, their IMS potency being confirmed, they were assayed in an *in vivo* transplantation experiment, based on Skin Grafting (SG), a method which can be appreciated as the direct correlate of human transplantation procedures.

Grafts vs. Host Reaction (GVHR).

Compounds **7**, **9** and **10** were evaluated utilizing a parenteral GVHR model modified by the Simonsen's technique.[21] The F-1 hybrid host animal is grafted with immunocompetent cells from a parent strain. The host spleen cells are incompatible to the grafted donor cells but are themselves not able to react against them (shared parental genetic determinants). The rejection mechanism in this model is therefore, from the graft toward the host. The grafted cells, introduced via intraperitoneal (i.p.) injection migrate to the host's spleen. There they proliferate in response to the difference in genetic determinants inherited from the other parental strain. The index used to

measure the success of the GVHR is splenomegaly (SI) (increased spleen weight due to cellular proliferation of grafted lymphocytes).

$$SI = \frac{[\text{spleen wt. of test group/body wt. of test group}] * 100}{[\text{spleen wt. of syngeneic group/body wt. of syngeneic group}]}$$

An index of 1.0 is equivalent to the spleen weight of syngeneic grafted controls. An index > 1.3 (graft index) is considered to be a successful graft rejection of the recipient animal. Any sample that prevents splenomegaly (< graft index) is considered as an active immunosuppressant. A high dose of cyclophosphamide (Cyp), a well established antineoplastic drug, was used for inducing great IMS and as standard for comparison purposes.

The results of such evaluations are shown in table 3 and it is noteworthy that the cyclolignans tested prevent splenomegaly at doses much more lower than that used for Cyp. Compound **10,** which was the most potent in the *in vitro* MLR assay, was also the most potent in the GVHR assay. Compounds **7** and **9** also gave promising results.

Table 3. *in vivo* GVHR Inmunosuppresive Activity Data for compounds 7, 9 and 10

Compound	Dose (mg/Kg) Inject	total	Body wt. Day 0 (g±SD)	Wt. change % Day 5	Spleen wt. normalized to D8 body wt.[a]	SI[b]	% Suppression[c]
7	15.0	105	16.5±0.5	1.8	6.34	1.27	---
	1.5	10.5	16.0±0.7	0.0	4.35	0.87*	148
	0.15	1.05	15.8±0.4	3.1	5.70	1.14	48
9	15.0	105	16.3±0.8	1.8	5.75	1.15	44
	1.5	10.5	16.8±1.6	2.9	5.20	1.04*	85
	0.15	1.05	16.5±0.9	-1.8	4.10	0.82*	167
10	15.0	105	16.3±0.8	-3.0	5.02	1.00*	100
	1.5	10.5	15.8±1.1	1.9	4.40	0.88*	144
	0.15	1.05	17.0±0.0	0.0	4.05	0.81*	170
Cyp	200	1400	16.5±0.5	-15.2	0.90	0.18*	404
SYN			15.7±0.9	6.7	5.00	1.00*	
POS			16.3±0.8	3.0	6.35	1.27	

[a] All treated animals survived the experiment and were sacrified on day 8. [b] SI: Spleen Index. see text for definition. [c] calculated with the equation (SI*pos* - SI*i*) *100/(SI*pos* - Si*syn*). * Significant activity: Index correlating to ≤50% suppression of positive control (≥1.3 expected)

Skin grafts.

Skin grafting (SG) was used to assess the altered T-cell function induced by immunosuppression active compounds identified by the MLR and GVHR techniques. In this assay an immunosuppressive compound is screened for its ability to prevent severe host-*versus*-graft disease due to allograft rejection. The model used in this procedure utilizes the same genetic strains of mice as in the GVHR method; namely, the Balb/c and CB6F-1 strains.[22]

After compounds **7, 9** and **10** proved their efficacy as immunosuppressant through the GVHR method, they were submitted to evaluation by the SG method.

CsA, the widest clinically used drug for preventing transplant rejection, at a dose of 25 mg/Kg, with the same schedule as for the lignan samples, was included in the assays for comparison. Table 4 contains the most relevant results and conditions for the experiments.

Table 4. Allogeneic Skin Grafting (SG) data for compounds 7, 9 and 10.

Compound	Dose[a] mg/Kg	Weight day 0 (g±SD)	Weight lost day 5	Mean graft survival (days±SD)	graft survival relative to: control	CyA
7	15	17.8±0.7	0.8	8.8±5.0	1.60	0.90
	1.5	18.7±1.2	1.7	6.8±3.2	1.24	0.69
	0.15	19.0±1.4	0.5	11.2±5.1	2.04	1.14
9	15	17.8±0.7	1.6	10.7±4.2	1.95	1.09
	1.5	18.5±1.0	1.0	12.5±5.4	2.27	1.28
	0.15	18.7±0.5	0.7	10.0±3.2	1.82	1.02
10	15	18.2±0.7	0.2	9.7±4.1	1.76	0.99
	1.5	18.5±0.8	1.0	11.0±3.7	2.00	1.12
	0.15	18.3±1.1	0.6	11.0±4.7	2.00	1.12
CsA	25	19.0±0.6	2.7	9.8±4.0	1.78	1.00
Control	-	18.3±0.8	-	5.5±1.1	1.00	0.56

a) ip. once a day for seven days. All animals were sacrified upon rejection or day 30. Those treated with lignans were alive after completion of the experiment (30 days). 20% of those treated with CsA died (mean survival time 14.8±0.0 days). All treated animals showed tolerance to grafting (≥ 150% of mean control graft survival).

Looking at the graft survival results several observations can be made. The three lignan derivatives tested, promoted tolerance to grafting beyond the control animals. The three compounds, at practically all doses produced significant prolongation (more than 50 %) of the survival time of grafting with respect to control. Compound **7** and **10** attained to maintain the graft for a time double that of the control, at low doses, while compound **9** gave still better results (2.3 times). All the three compounds, at comparatively lower doses, gave better results than CsA. Furthermore no deaths occurred during the time of the experiments within the groups of animals treated with lignans, whereas 20% of those treated with CsA died (mean survival time 14.8 ± 0.0 days). Weight lost was also significantly lesser for those animals treated with lignans.

Of the structural modifications performed on the molecule of podophyllotoxin and its congeners, the demethylenation of dioxol grouping and the aperture of the γ-lactone ring, with simultaneous formation of a fused isoxazole heterocycle, seem to be adequate transformations for retaining, or even increasing, the immunosuppressive activity of cyclolignans. Further work is needed to establish their mechanism of action, in which the phenolic groups at C-3 and/or C-4 could play a prominent role and hence, most probably will be different of the two well accepted mechanism for cyclolignans as antineoplastic: the inhibition of tubulin polymerization and inhibition of DNA topoisomerase II.[14b] In spite of the small number of molecules and although the evaluation of these type of compounds is practically at the beginning, it can be stated that natural cyclolignans, upon the adequate structural manipulation, have a great potentiality for being considered as candidates for the development of clinically useful immunosuppressants.

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22. Skin grafts were prepared by dermal punches (1-2 cm., diameter) from the skin of a sacrificed male CB6-F1 mice ($H2^d + H2^b$). These were placed into a solution of normal saline until further use. The grafts were applied at 180 °C of their original orientation to the dermal punched backs of anesthetized (90 mg/kg ketamine·HCl and 10 mg/kg rompun) recipient male Balb/c ($H2^d$) mice. A liquid bandage was applied to the grafted area of the recipient animal by using an aerosol form of a Collodion (containing no clove extracts of other fragrances)/isopropanol solution. On D1 (> 16 hours), groups of individually caged animals (5/group) received an i.p. injection *per diem* of an active dose of test compounds, as previously determined in the GVHR, for as long as the graft survived up to 30 days. Grafts that slough-off on or before D3 post transplantation were considered 'no takes'. Normal graft rejection in control animals occured between day 5-6 post transplantation. Beyond D6, evaluation of the grafted skin was graded daily for the following characteristics: erythema, crusting, purulent discharges, sensation of the grafted skin and status of the hair (occuring in prolonged graft survival studies). 'No takes', control animals post D6 and moribund animals were sacrificed. Experimental animals were sacrificed on the day of graft rejection or D30, whichever came first.

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