Immunosuppressive Cyclolignans

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The immunosuppressive activity of several lactonic, nonlactonic, and heterocycle-fused cyclolignans has been demonstrated for the first time by use of a T-cell-mediated immune response. Of the compounds tested, 4′-demethyldeoxypodophyllotoxin (**8**), *β*-apopicropodophyllin (**6**), and the isoxazoline-fused cyclolignan **15** are the most potent with respect to their suppression of activated splenocytes.

Introduction

Immunomodulators are natural or synthetic products capable of modifying the normal or aberrant immune system through stimulation or suppression. Currently, they constitute an important category of therapeutic agents in a rather wide range of conditions from allergy and anaphylaxis to psoriasis, rheumatoid arthritis, and cancer.[1] Compounds with established immunomodulatory properties have discrete or polymeric structures, such as steroids, purine derivatives, polypeptides, and glycosides. As a common feature, most of these materials have inherent toxicity and adverse side effects which direct the search for new and safer agents. Consequently, apart from the demonstration of their therapeutic efficacy, the search for new useful immunosuppressants is mainly guided by the absence of cytotoxicity and other adverse side effects of these putative drugs.[2]

In common with the majority of chemotherapeutics used against malignancy, AIDS, and other viral diseases, lignan derivatives such as etoposide, teniposide, and podophyllotoxin have a number of undesirable side effects (anemia, alopecia, carcinogenicity, gastrointestinal disturbances, etc.). These agents also diminish the natural defenses and immune responses of treated patients, making them susceptible to opportunistic attacks by bacteria, fungi, and other microorganisms. This immunosuppressive effect can be put to good use as it can be used to prevent the acute rejection of transplanted organs. Thus, the alkylating antineoplastic cyclophosphamide, the macrolide FK-506, the purine antimetabolite azathioprine, and, principally, the polypeptide antibiotic cyclosporine A (CyA), although cytotoxic immunosuppressants, are used clinically to prolong the survival of transplanted organs.[2]

During the past few years, we had the aim of finding more potent and/or less toxic antineoplastic compounds and we prepared a considerable number of cyclolignans related to podophyllotoxin. One of the main conclusions of our qualitative SAR analysis was that the presence of the *trans*-fused *γ*-lactone is a very important feature in relation with the cytotoxicity levels of this type of lignan.[3,4] Recently we have reported some chemical results which permitted us to propose a mechanistic explanation for the irreversible interaction of the podophyllotoxin type of lignan and receptor biomolecules.[5]

As a continuation of our research in this area, a number of lignan derivatives, which are representative of several classes of natural and semisynthetic cyclolignans related to podophyllotoxin and *β*-peltatin, have been subjected to *in vitro* evaluation of their immunomodulatory (IM) activity. As the standard for characterizing immunossupression CyA was used.

Chemistry

Some of the compounds tested or used as starting materials were isolated from *Podophyllum* resin by means of chromatographic procedures; that is the case of podophyllotoxin (**1**), deoxypodophyllotoxin (**2**), *β*-peltatin (**3**), and α-peltatin (**4**) (Scheme 1). **1** was transformed into **5**,[6] **6**,[7] and **7**[8] by described procedures.

Compounds **8**−**11** have a phenolic hydroxyl group instead a methoxyl group at position C-4′ of the phenyl ring. They were obtained from the corresponding trimethoxyphenyl analogues by demethylation with HBr−AcOH 33% and futher acetylation in the case of **9** and **10**.

The heterocyclic-fused cyclolignans **12**−**14** were prepared from podophyllotoxone (**7**). **7** was obtained from **1** by oxidation with pyridinium dichromate and then condensed with the appropriate hydrazine derivative in glacial acetic acid at room temperature. The resulting carboxylic acid was transformed into the corresponding derivatives **12**−**14** as described previously.[5] Similarly, by reaction of **7** with hydroxylamine in ethanol, compound **15** was obtained.

Compound **16** was obtained from **1** after opening the lactone ring in basic conditions, followed by oxidation of the benzylic hydroxyl group with Jones' reagent at 0 °C.

Biological Results and Discussion

The method used for IM evaluation, the mixed lymphocyte reaction (MLR) method, is a radioisotopic assay involving cell mediated immune reactions.[9] This is accomplished using a two-way MLR derived from murine splenocytes of genetically dissimilar strains of mice. The MLR data are calculated as a percentage of immune cell proliferative activity relative to control, and IC_{50} values are interpolated for each test compound. In parallel, the cytotoxic properties of the samples in evaluation are established by the LcV (lymphocyte viability) assay[10] and their IC_{50} values interpolated from the cell viability percentages relative to control. MLR

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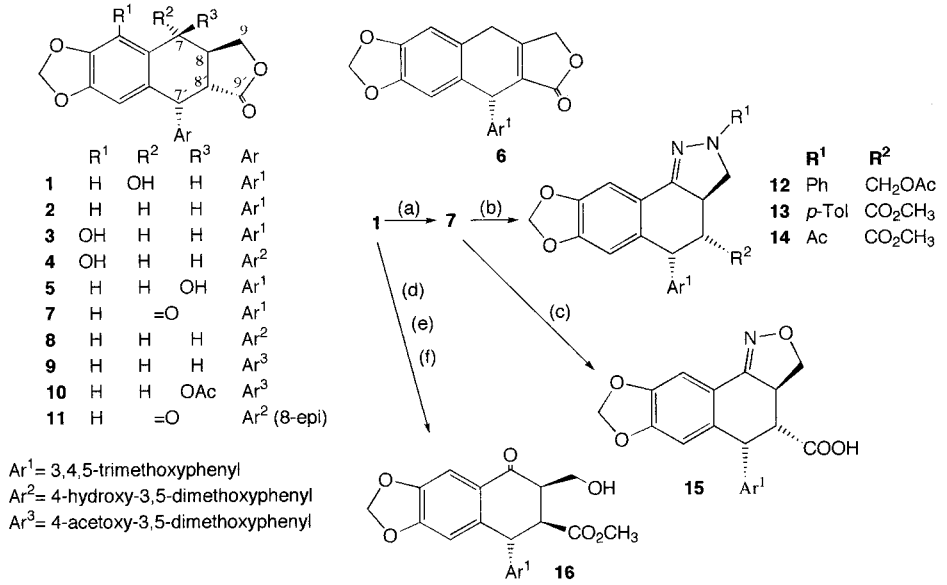
Scheme 1[a]



[a] (a) Pyridinium dichromate, Cl_2CH_2 room temperature; (b) H_2N-NHR_1, AcOH, room temperature; (c) H_2NOH, EtOH−Py, room temperature; (d) KOH/MeOH 1%, room temperature; (e) CH_2N_2−ether; (f) CrO_3, H_2SO_4, acetone, 0 °C.

Table 1. Immunosuppressive Activity and Cytotoxicity Data for Several Cyclolignan Derivatives

compd	MLR[a]	LcV[b]	nCIMI[c]
1	<0.128	280	>2190
3	<0.00945	19.7	>2085
4	<0.00495	12.8	>2586
5	0.229	>65.0	>284
6	0.00825	27.5	>3333
8	0.00281	11.5	4097
9	0.0718	15.8	220
10	0.453	22.8	50
11	11.8	>25.0	>2
12	9.29	>100	>11
13	0.848	>26.0	>31
14	7.68	>100	>13
15	<0.00900	30.0	>3333
16	1.41	>25.0	>18
CyA	0.0518	2.75	53

[a] IM activity, IC_{50} (μg/mL). [b] Cytotoxic activity, IC_{50} (μg/mL). [c] Noncytotoxic IM activity (ratio LcV/MLR).

and LcV values are then compared to provide a measure of the noncytotoxic immunosuppressive activity (nCIM Index, nCIMI) for each substance.

From the MLR data in Table 1, considered separately and within the non-nitrogenated derivatives, it can be seen that the most potent IM compounds, which are in the order **8** > **4** > **6** > **3**, have no substituent at position C-7. Thus, a reasonable analogy between IM and antineoplastic activities[3,4] is observed. The potency for compounds having an oxygenated function at the same position decreases in the order α-OH (**1**) > β-OH (**5**) > OAc (**10**) > CO (**11**). The opening of the lactone fragment, as in compound **16**, also decreases the IM potency.

Another structural feature that could be important for the activity is the nature of the substitution on the aryl ring at the C-4′ position. Compounds **4** and **8**, which have a free phenolic hydroxyl at the 4′-position of the phenyl ring, were more potent than the corresponding compounds **3** and **6**, which have a methoxy group at that position. In addition, acetylation of the phenolic hydroxyl in **8** afforded **9**, which proved to be about 20 times less potent than **8**. A free phenolic group on the C-4′ phenyl ring has also been observed to be important for the inhibitory activity of 7-epipodophyllotoxin derivatives against DNA topoisomerase-II.[6]

Compounds having a pyrazoline ring fused to the cyclolignan are among the least potent of the series. This decrease could be due either to the presence of the substituent R^1 attached to the saturated nitrogen atom or to the methylation of the carboxyl group, because the oxazoline analog **15** is as potent as those lignans having the γ-lactone ring.

All the compounds exhibited similar activities in the LcV assay within a 10-fold range. Therefore the comparison of relative potency of these cyclolignan derivatives as immunosuppressants, through their noncytotoxic IM activity indexes (nCIMI), follows a pattern similar to that found when the MLR results were considered alone. However, while 4′-demethyldeoxypodophyllotoxin (**8**) was the most potent immunosuppressive compound of the series, β-apopicropodophyllin (**6**) and the isoxazoline derivative (**15**) showed better nCIMI than **3** and **4** and as a result are better candidates for further *in vivo* evaluation.

The most potent of the immunossupressive activities of the cyclolignans tested occurred with IC_{50} values between 3 and 9 ng/mL, wich could be consider in the same range of CyA (51.8 ng/mL). However, CyA is cytotoxic, thereby effectively reducing its therapeutic index (nCIMI of 53) compared to the cyclolignans (nCIMI ranging 2000−3000). In conclusion, it can be reported that several cyclolignans (**1**,**3**, **4**, **6**, **8**, and **15**) may act to suppress a T-cell-mediated immune response by a noncytotoxic mechanism of action.

Although the number of compounds tested is small, from these preliminary results, it could be concluded that the chemical transformation of podophyllotoxin-related cyclolignans has led to several derivatives which show promising IM activity that merit further attention. This is the first report of direct experimental evaluation of this property in cyclolignans, although there are some references to the potential utility of lignans.[11,12]

Experimental Section

Biological Assays. Mixed Lymphocyte Reaction (MLR). Each compound was solved in absolute EtOH, and eight dilutions (concentrations from 10 to 3.33×10^{-6} mg/mL) were prepared. Duplicate volumes (10 mL) of each dilution were added into the wells of a 96-well microtiter plate and then evaporated to dryness at room temperature. Controls were prepared similarly. Splenocytes from Balb/c (H-2^d) and C57Bl/6 (H-2^b) mice (100 mL of each cell suspension) were added together to test and control wells. Negative control wells were prepared by adding only 200 mL of each splenocyte suspension and nonspecific control wells by adding only 200 mL of culture media. Plates were incubated in a 5% CO_2-humidified incubator at 37 °C for 96 h and then pulsed for 15 h with 1 mCi of [^{3}H]thymidine (20 Ci/mmol) per well. The contents of each well were filtered through a glass fiber strip and the tritiated-thymidine incorporated into newly synthesized DNA measured using a dry scintillant counter.

Lymphocytes Viability (LcV) Assay. A 200 mL portion of Balb/c splenocyte suspension was added to one set of test compounds and control wells. A 200 mL sample of culture media was added to another set of compounds to serve as nonspecific control. The plates were incubated as above, then pulsed with 75 mL/well of MTT-thiazolyl blue solution (150 mg), and decanted. The resulting insoluble formazan crystals, if formed, were dissolved in 200 mL of 2-propanol and read at 570 nm with a plate reader.

Chemistry: General Procedures. Melting points were determined by heating in an external silicone bath and were uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter in chloroform solution and UV spectra on a Hitachi 100-60 spectrophotometer in ethanol solution. IR spectra were obtained on a Beckmann (Acculab VIII) spectrophotometer in chloroform solution. EIMS were run in a VG-TS-250 spectrometer working at 70 eV. NMR spectra were recorded at 200 MHz for ^{1}H and 50.3 for ^{13}C in deuteriochloroform using TMS as internal reference, on a Bruker WP 200 SY. Chemical shift values are expressed in ppm followed by multiplicity and coupling constants (*J*) in hertz. Flash chromatography was performed on silica gel (Merck No 9385). Elemental analysis were carried out on a Perkin-Elmer 2400 CHN elemental analyzer.

Isolation of Lignans 1, 2, 3, and 4. The resin (50 g) of *Podophyllum peltatum* was extracted with hot $CHCl_3$. The soluble fraction was chromatographed on neutral alumina (activity II), and the following cyclolignans were eluted with $CHCl_3$: deoxypodophyllotoxin (**2**, 1%), podophyllotoxin (**1**, 8%), β-peltatin (**3**, 9%), and α-peltatin (**4**, 7%).

4′-Demethyldeoxypodophyllotoxin (8). HBr−AcOH 33% (2.5 mL) was added to a solution of **2** (208 mg) in 1,2-dichloroethane (10 mL), and the mixture was stirred for 15 h at room temperature. The reaction mixture was added to a saturated solution of $NaHCO_3$ mixed with ice and extracted with EtOAc. The extract was washed with brine, and dried over Na_2SO_4, and the solvent was evaporated off. The reaction product was chromatographed to yield 155 mg of **8** (72%). Physical data is in accord with those described previously.[13] Acetylation of **8** with acetic anhydride in pyridine afforded **9**.

Similarly, the following 4′-demethylated cyclolignans were obtained.

4′-Demethylisopicropophyllone (11): from **7** (46%). Physical data is in accord with those described previously.[13]

4′-Demethylepipodophyllotoxin Diacetate (10): from **1** (49%) after acetylation and flash chromatography of the reaction product. Physical data is according with those described previously.[6]

Isoxazopodophyllic Acid (15): Pyridine (0.2 mL) and hydroxylamine chlorhydrate (64 mg, 0.93 mmol) were added to a solution of **7** (300 mg, 0.73 mmol) 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 **15** were separated after crystallization with CH_2Cl_2: mp 246−248 °C; MS *m*/*z* 483 (M^+), 329, 286, 260, 176, 133, 89; $[\alpha]^{22}_D$ −152.6° (*c* 0.6, $CHCl_3$); IR (cm^{-1}, $CHCl_3$) 3400−2800, 1710, 1600, 1510, 1490, 1470, 1230, 1040, 940, 880, 860; UV (ϵ) (nm, EtOH) 215 (26 500), 274 (1200), 313 (8900); ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.38 (1H, bs), 7.43 (1H, s, H-2), 6.56 (1H, s, H-5), 6.20 (2H, s, H-2′,6′), 5.99 (2H, s, OCH_2O), 4.82 (1H, t, J = 12.3 Hz, H-9a), 4.67 (1H, d, J = 7.0 Hz, H-7′), 3.80 (2H, m, H-8, H-9b), 3.76 (3H, s, MeO-4′), 3.66 (6H, s, MeO-3′, 5′), 3.23 (1H, dd, J = 11.4 and 5.0 Hz, H-8′); ^{13}C NMR (50.3 MHz, CD_3OD) δ 178.8 (C-9′), 159.5 (C-7), 154.0 (C-3′,5′), 151.9 (C-4), 148.8 (C-3), 138.5 (C-6), 138.2 (C-1′), 138.0 (C-4′), 120.2 (C-1), 110.4 (C-5), 109.2 (C-2′,6′), 104.2 (C-2), 103.1 (OCH_2O), 76.2 (C-9), 61.0 (MeO-4′), 56.4 (MeO-3′,5′), 53.8 (C-8′), 49.0 (C-7′), 46.0 (C-8). Anal. ($C_{22}H_{21}O_8N$) C, H, N.

Methyl 7-Oxodeoxypicropodophyllate (16): 1 (1000 mg) was added to a solution of 5% KOH/MeOH and stirred for 30 min at room temperature. After partial evaporation of the solvent, water and HCl (2 N) until pH = 5 were added and extracted with EtOAc. The reaction product was treated with an ethereal solution of CH_2N_2 to yield the corresponding dihydroxy ester (90%). Jones' reagent (1 mL) was added to a solution of the dihydroxy ester (220 mg) in acetone (4 mL) and stirred at 0 °C for 90 min. Excess oxidant was destroyed by addition of saturated sodium bisulfite solution. The organic layer was diluted with EtOAc, washed with brine, and dried over Na_2SO_4, and the solvent was evaporated *in vacuo*. The reaction product was chromatographed to afford compound **16** (50%): mp 76−78 °C (H/EtOAc); $[\alpha]^{22}_D$ −46.8° (*c* 1, $CHCl_3$); IR (cm^{-1}, $CHCl_3$) 3500, 1740, 1680, 1600, 1510, 1490, 1470, 1260, 1045, 945, 895, 850. UV (ϵ) (nm, EtOH) 212 (12 000), 233 (12 000), 273 (7600), 314 (6300); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.55 (1H, s, H-2), 6.53 (1H, s, H-5), 6.24 (2H, s, H-2′,6′), 6.06 (1H, s, OCH_2O), 6.03 (1H, s, OCH_2O), 4.62 (1H, d, J = 2.8 Hz, H-7′), 4.24 (1H, dd, J = 11.2 and 7.8 Hz, H-9a), 3.83 (3H, s, MeO-4′), 3.77 (6H, s, MeO-3′,5′), 3.65 (3H, s, COO*Me*), 3.58 (1H, dd, J = 11.2 and 4.4 Hz, H-9b), 3.28 (1H, dd, J = 2.8 and 3.8 Hz, H-8′), 2.95 (1H, m, H-8); ^{13}C NMR (50.3 MHz, CD_3OD) δ 188.8 (C-7), 172.7 (C-9′), 153.7 (C-3′,5′), 152.8 (C-4), 147.7 (C-3), 137.9 (C-4′), 137.8 (C-1′), 137.1 (C-6), 127.8 (C-1), 109.3 (C-5), 106.6 (C-2′,6′), 105.7 (C-2), 101.9 (OCH_2O), 62.1 (C-9), 60.7 (MeO-4′), 56.5 (MeO-3′,5′), 52.1 (COO*Me*), 50.9 (C-8′), 47.8 (C-7′), 45.9 (C-8).

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