If the drug participates in a chemical reaction in the compartment fluid phase of the physiological system, such effects may contribute to either alteration of corresponding element of vector X of Eq. 1 or, in principle, change an n-compartment model to behave as an $(n + 1)$-compartment model. These considerations will be discussed in future publications; for the present, it is concluded that such a phenomenon is capable of possibly explaining certain types of "aperiodic biological oscillatory" after effects observed with certain drugs in physiological systems.

APPENDIX

Defining the quantities ϵ and ζ by:

$$\epsilon = \alpha_{12} + (\delta_{13}/V_1) \quad \text{(Eq. A1}a\text{)}$$

$$\zeta = \epsilon + k_{20} \quad \text{(Eq. A1}b\text{)}$$

one has:

$$\omega = k_{20} - \epsilon \quad \text{(Eq. A2)}$$

Equations 22a–22c of Ref. 1 can now be expressed as:

$$C_1(J_1/\eta) = -(\mu_3 + \mu_2) - \zeta \quad \text{(Eq. A3}a\text{)}$$

$$C_2(J_2/\eta) = (\mu_1 + \mu_3) + \zeta \quad \text{(Eq. A3}b\text{)}$$

$$C_3(J_3/\eta) = -(\mu_1 + \mu_2) - \zeta \quad \text{(Eq. A3}c\text{)}$$

$$J_1 = (\mu_1 - \mu_2)(\mu_1 - \mu_3) \quad \text{(Eq. A3}d\text{)}$$

$$J_2 = (\mu_1 - \mu_2)(\mu_2 - \mu_3) \quad \text{(Eq. A3}e\text{)}$$

$$J_3 = (\mu_1 - \mu_3)(\mu_2 - \mu_3) \quad \text{(Eq. A3}f\text{)}$$

The J_σ's, η, and ζ are positive definite. The decay constants are distinct and are negative definite. They may be ordered such that their magnitudes satisfy the inequalities of $|\mu_1| > |\mu_2| > |\mu_3|$.

It is evident that both C_1 and C_3 are negative when the magnitude of ζ is greater than $-(\mu_1 + \mu_2)$ [greater than $-(\mu_2 + \mu_3)$]. Because of the constraint expressed by Eq. 21b of Ref. 1, C_2 should be positive definite, which is assured by Eq. A3b.

The condition imposed by Eq. 12 for the existence of t_1^* is that the ratio $(1 + r_3m)/(1 + r_2m)$ should be positive. This condition is evidently satisfied if the roots of cubic Eq. 19 of Ref. 1, r_2 and r_3, are both positive and have a magnitude less than k_{20}. This condition is also satisfied if both r_2 and r_3 are negative, provided the magnitudes of r_2m and r_3m are greater than unity. It is also satisfied if either r_2 or r_3 is negative, provided the product of negative root and m has a magnitude less than unity.

When α_{13} is greater than k_{20}, from Eq. 17c of Ref. 1 it follows that $R = -(r_1r_2r_3)$ is negative. This requires that, when r_1 is negative, r_2 and r_3 must have opposite signs.

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Potential Anticancer Agents II: Antitumor and Cytotoxic Lignans from *Linum album* (Linaceae)

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Abstract □ A phytochemical study of *Linum album* (Linaceae), guided by bioassay with the 9KB cell culture, resulted in the isolation of podophyllotoxin and a new lignan, 3′-demethylpodophyllotoxin; α- and β-peltatins were identified by comparative TLC.

Keyphrases □ *Linum album* (Linaceae)—isolation and identification of antitumor and cytotoxic lignans □ Lignans—isolation and identification from *L. album*, screened for anticancer and cytotoxic properties □ Anticancer agents, potential—isolation and identification of antitumor and cytotoxic lignans from *L. album*, screened for activity

Linum album was found to be active against the P-388 leukemia and 9KB cell assay system in a random collection screening program for new anticancer agents. The chloroform extract of *L. album* yielded podophyllotoxin, α- and β-peltatins, and a new lignan, 3′-demethylpodophyllotoxin. Based upon spectroscopic data, a structure was proposed for 3′-demethylpodophyllotoxin, which was verified by preparation of a derivative of known structure. The lignans podophyllotoxin, α- and β-peltatins, and 3′-demethylpodophyllotoxin were shown to be at least partially responsible for the antitumor and cytotoxic activity of *L. album* extracts.

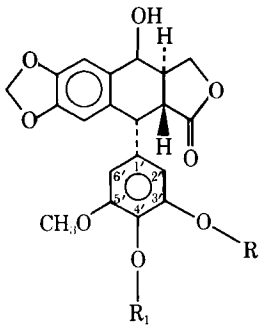
EXPERIMENTAL[1]

Biological Activity—An ethanolic extract of *L. album* was evaluated for cytotoxicity; it was found active against Eagle's 9KB carcinoma of the nasopharynx in cell culture (ED_{50} = 2.3, 2.5, and 1.4 μg/ml)[2] and showed *in vivo* activity against the P-388 lymphocytic leukemia in mice (T/C 136, 126, and 108 at 133, 88, and 88 mg/kg, respectively)[3]. Test methods employed were those of the Drug Research and Development Branch of the National Cancer Institute (1).

[1] The plant material used in this investigation consisted of the whole plant of *L. album* Kotschy ex Boiss. (Linaceae), collected in Iran during July 1970 and provided by Dr. T. Fakouhi, Department of Pharmacology, Pahlavi University, Medical School, Shiraz, Iran. Voucher specimens were identified by R. E. Perdue and are deposited in the Herbarium of the U.S. Department of Agriculture, Beltsville, Md.

[2] An active fraction is one that exhibits an $ED_{50} \leq 20$ μg/ml.

[3] An active fraction is one that exhibits a T/C of ≧125%.



lignan	R	R_1
I	CH_3	CH_3
II	CH_3	OH
III	OH	CH_3

Fractionation of the initial plant material into Fractions A, C, and D (Scheme I) showed that the activity was mainly concentrated in Fraction A (1.8 μg/ml in the 9KB system and T/C values in mice infected with the P-388 leukemia of 130, 125, and 125 at doses of 400, 200, and 100 mg/kg, respectively). Fraction C was cytotoxic (ED_{50} = 1.4 μg/ml) as well as toxic in mice infected with the P-388 leukemia at doses of 100–400 mg/kg. Fraction D was inactive in the 9KB and P-388 systems. These data are presented in Table I.

Fractionation of Plant Material—A sample of whole plant material (6.6 kg) was continuously extracted in a Lloyd extractor with petroleum ether (bp 30–60°) until the extract was colorless. This extract was then concentrated to a viscous, light-brown syrup weighing 82 g (Fraction A). The defatted plant material was air dried and continuously extracted with methanol (3 liters), and the methanol solution was concentrated *in vacuo* to yield 390 g of a thick, dark-brown syrup (Fraction B).

Fraction B was partitioned between chloroform (6 liters) and water (3 liters). The chloroform fraction (Fraction C) was separated, dried over anhydrous sodium sulfate, and concentrated to dryness *in vacuo* to give a residue of 107 g. The aqueous phase was frozen and lyophilized to yield a residue weighing 280 g (Fraction D).

Fractionation of Fraction C—The chloroform-soluble fraction (C, 100 g) was dissolved in methanol and intimately mixed with 250 g of silica gel PF_{254}. Following evaporation of the methanol *in vacuo*, the powdered mixture was slowly added to the top of a chloroform slurry-packed column containing 3.0 kg of silica gel PF_{254}. Elution was initiated with chloroform, and 4.0-liter fractions were collected.

The eluent was changed to solvents of increasing polarity as the fractions were monitored by spraying the developed plates with sulfuric acid and heating at 120° for 10 min. The fractions were combined into five groups having different TLC patterns and were subsequently assayed for 9KB and P-388 leukemia activity (Table II).

Table I—Antitumor and Cytotoxic Screening Data on *L. album*

Extract	Fraction	Tumor Systems: 9KB ED_{50}, μg/ml[a]	Tumor Systems: P-388 Dose/Percent[b], (mg/kg)/(T/C)
50% ethanol	B001	2.3	133/136
		2.5	88/126
		1.4	
50% ethanol	B002	1.4	88/108
	A	1.8	400/130
			200/125
			100/125
	C	1.4	400/TT[c]
			200/TT
			100/TT
	D	100.0	400/95
			200/110
			100/110

[a] An active fraction is one that exhibits an $ED_{50} \leq 20.0$ μg/ml. [b] An active fraction is one that exhibits an increase in survival time ≥125%. [c] TT = too toxic.

Table II—Chromatographic Separation of Fraction C

Fractions[a]	Eluent	Weight, g	9KB[b] ED_{50}, μg/ml	P-388[c], Dose/Percent, (mg/kg)/(T/C)
1–4	Chloroform	1.79	100.0	130/90
5	Chloroform	16.2	12.0	100/135
6	Chloroform	20.8	6.9	100/135
7	Chloroform	2.4	6.0	140/140
8–13	Chloroform–methanol (1:1)	13.0	79.0	400/135

[a] Each fraction was 4.0 liters. [b] An active fraction is one that exhibits an $ED_{50} \leq 20$ μg/ml. [c] An active fraction is one that exhibits an increase in survival time ≥125%.

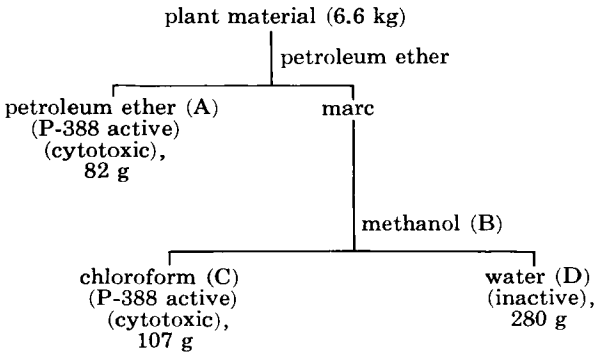
Detection of α-Peltatin and β-Peltatin—A 55-mg sample derived from fraction 6 (Table II) gave a strong positive phenol test with diazotized *p*-nitroaniline spray reagent (2). To remove the phenolic constituents from this mixture, the sample was dissolved in ether (10 ml) and extracted with 10 ml of 10% sodium carbonate solution. The sodium carbonate solution was then acidified with concentrated hydrochloric acid, and the acidic solution was extracted with ethyl acetate.

After drying the organic phase over anhydrous sodium sulfate, it was filtered and evaporated to dryness *in vacuo* to yield a residue (31 mg). TLC analysis of this sample indicated that it was a complex mixture of phenolic compounds. Co-TLC with reference α-peltatin and β-peltatin revealed the presence of these two compounds in the sample[4]. Both peltatins gave a reddish color when sprayed with diazotized *p*-nitroaniline.

α-Peltatin gave an R_f value of 0.30 in benzene–95% ethanol (9:1), 0.20 in benzene–chloroform–methanol (10:5:0.5), and 0.10 in methylene chloride–ethyl acetate (5:1); β-peltatin gave R_f values of 0.40, 0.25, and 0.15, respectively, in these three solvent systems. The complexity of the sample and the small quantity precluded further purification.

Isolation of Podophyllotoxin—Fraction 6 (Table II) was further chromatographed to yield several subfractions, which contained podophyllotoxin (I) as indicated by co-TLC with reference podophyllotoxin[4]. However, these combined samples (278 mg) proved complex and exhibited a green color. These subfractions were partitioned between petroleum ether and 10% aqueous methanol in an attempt to simplify the mixture. Only the aqueous methanol fraction showed the presence of podophyllotoxin.

The residue (220 mg), after evaporation of methanol, was dissolved in benzene (2 ml) and chromatographed[5]. Elution with 1



Scheme I

[4] Supplied by Dr. J. L. Hartwell, Drug Research and Development, National Cancer Institute, Bethesda, Md.

[5] Twenty grams of Florisil F-101 packed in benzene, Fisher Scientific Co., Fair Lawn, N.J.

Table III—NMR Spectra of 3′-Demethylpodophyllotoxin[a]

Proton Assignment	Chemical Shift, δ, ppm	Multiplicity, J, Hz (in $CDCl_3$)	Integration	Chemical Shift, δ, ppm	Multiplicity, J, Hz (in Benzene-d_6)	Integration
Aliphatic	2.75–3.55	Multiplet	2	—	—	—
Ar-OCH_3	3.82	Singlet	6	3.55	Singlet	3
				3.58	Singlet	3
Aliphatic	4.55	Multiplet	2	—	—	—
Methylenedioxy	5.99	Singlet	2	—	—	—
Aromatic	6.01	Doublet (J = 2 Hz)	1	6.28	Doublet (J = 2 Hz)	1
Aromatic	6.45	Singlet	1	6.40	Singlet	1
Aromatic	6.78	Doublet (J = 2 Hz)	1	6.97	Doublet (J = 2 Hz)	1
Aromatic	7.18	Singlet	1	7.10	Singlet	1

[a] The spectra were recorded at 60 MHz.

liter of benzene removed the green color. Subsequent elution with chloroform (1 liter) and evaporation of the eluant *in vacuo* afforded a pale-yellow residue (154 mg). Crystallization and recrystallization of this material, using benzene–petroleum ether, yielded white crystals (30 mg), mp 115–120°.

A UV[6] spectrum exhibited absorption with λ_{max} at 292 nm (log ε 3.29), and bands in the IR spectrum[7] (KBr) at ν_{max} 3400 (OH) and 1750 (lactone) cm^{-1} were observed. The mass spectrum[8] showed a molecular ion at *m/e* 414 (100%). The IR, mass spectral, and TLC properties were identical with those of podophyllotoxin (3–5), and there was no depression of the melting point following a mixed melting-point determination. The absolute configuration of I has recently been determined by X-ray analysis (6).

Isolation and Characterization of 3′-Demethylpodophyllotoxin—Fraction 6 from the first silica gel column (Table II) gave a positive test with diazotized *p*-nitroaniline spray reagent (2). Subsequent purification of this fraction, using another silica gel PF_{254} column, afforded a semipurified material (99 mg) containing a phenolic component. The sample was dissolved in chloroform (1 ml), applied to a 2.0-mm thick silica gel PF_{254} plate, and developed with ethyl acetate–petroleum ether (9:1).

The zone corresponding to R_f 0.70 was removed, eluted with methanol, and filtered, and the filtrate was taken to dryness *in vacuo*. The resulting pale-yellow amorphous solid (30 mg), mp 85–105°, gave a UV absorption with λ_{max} at 289 nm (log ε 3.30), indicative of a podophyllin derivative (5); important bands in the IR (KBr) spectrum were at ν_{max} 3400 (OH), 1760 (lactone), and 1590 (aromatic) cm^{-1}. TLC of the isolate on silica gel G gave R_f 0.65 using 1-butanol–acetic acid–water (4:1:1) and R_f 0.25 using ethyl acetate–methanol (95:5). The color produced with diazotized *p*-nitroaniline spray reagent was bright orange.

The mass spectrum of this isolate showed a molecular ion at *m/e* 400 (100%), and the fragmentation pattern was characteristic of that produced by lignan derivatives (3, 4). The loss of water (M^+ − 18) (5.0%) indicated that an alcohol group was present, and fragments at *m/e* 154 (23%) and *m/e* 167 (16%) indicated the presence of a phenolic group in ring D in addition to two methoxyl groups (Scheme II). This suggested that the isolate might be identical with the known lignan 4′-demethylpodophyllotoxin (II). However, co-TLC and mixed melting-point determination with an authentic sample of 4′-demethylpodophyllotoxin eliminated this possibility[9].

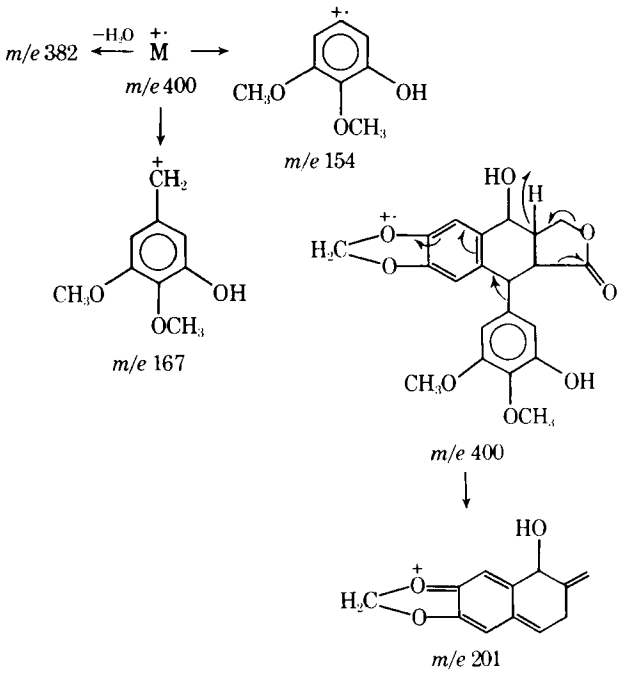
The NMR spectrum[10] in $CDCl_3$ (Table III) confirmed the presence of two methoxyl groups, exhibiting a singlet integrating for six protons at δ 3.82. Another singlet was also observed, and the chemical shift of δ 5.99, together with the integration for two protons, suggested a methylenedioxy group. Comparison of this spectrum with that of II[9] indicated that only the aromatic region was different.

In the NMR spectrum of II, the presence of a singlet at δ 6.42, integrating for two protons, was representative of the equivalent 2′ and 6′ aromatic protons. No such absorption was observed for the isolate, but a pair of doublets (J = 2 Hz), each integrating for one proton, was seen at δ 6.78 and 6.01. The absorption at δ 6.01 was somewhat obscured by the methylenedioxy peak at δ 5.99. Irradiation at δ 6.01 collapsed the doublet at δ 6.78 to a singlet. Back-irradiation produced a similar result for the doublet at δ 6.01.

An NMR spectrum was taken using benzene-d_6 (Table II) as the solvent, with the hope of observing solvent shifts of the aromatic and the methoxyl protons. Indeed, the methoxyl protons were shifted and split into two singlets at δ 3.55 and 3.58, indicating that the methoxyl groups were nonequivalent; the fact that they appeared as a singlet at δ 3.82 in the spectrum taken in $CDCl_3$ could now be attributed to its characteristic in the solvent. Furthermore, the aromatic protons were shifted so that the two doublets were observed at δ 6.28 (J = 2 Hz) and 6.97 (J = 2 Hz). A double-resonance experiment again indicated that these two protons were coupled to each other.

These data indicated that the two aromatic protons in ring D were nonequivalent and *meta* (J = 2 Hz) to each other and also that the two methoxyl groups were nonequivalent. At this point, only five possible arrangements of the three substituents (*i.e.*, two methoxyl and one OH) on ring D could be considered.

Only one of these possible compounds would give rise to podophyllotoxin. Methylation of the isolate with diazomethane did in-



Scheme II—Mass spectral fragmentation of 3′-demethylpodophyllotoxin

[6] Recorded using a Beckman model DB-G grating UV spectrophotometer.
[7] Recorded using a Beckman model IR-18A IR spectrophotometer.
[8] Recorded using a Hitachi Perkin-Elmer model RMU-6D mass spectrometer.
[9] Supplied by Sandoz Inc., Basel, Switzerland.
[10] Recorded using a Varian T-60A instrument at 60 MHz.

deed yield podophyllotoxin, hence proving that the isolate was 3′-demethylpodophyllotoxin (III).

No differences were observed in the NMR spectra of the aliphatic regions of I, II, or III, indicating that III has the same stereochemistry at the corresponding positions as I. This is the first report of the isolation of III from nature.

DISCUSSION

Podophyllotoxin (I) was reported previously to be cytotoxic in the 9KB system, with an ED_{50} of <0.01 μg/ml (7); it is active in the Lewis lung (LL) carcinoma system (mice) at a minimum effective dose (MED) of 2 mg/kg, giving a tumor weight inhibition of 64% (7). Furthermore, it is active in the P-388 leukemia system with an MED of ~2 mg/kg, producing a 70% increase in lifespan (7). This cytotoxic lignan is also active in the Walker 256 (IM) carcinosarcoma system; tumor weight is inhibited 73% at an MED of 6 mg/kg.

However, podophyllotoxin has been reported to be inactive in the following tumor systems: sarcoma 180, adenocarcinoma 755, L-1210 leukemia (intraperitoneal), Friend virus leukemia, melanotic melanoma, plasmacytoma No. 1, HS1 human sarcoma, L-1210 leukemia (subcutaneous, delayed treatment), and P-1798 lymphosarcoma (7).

Other workers (8) stated that podophyllotoxin is effective against the L-1210 leukemia, lymphosarcoma, lymphocarcoma 2, mammary adenocarcinoma C3HBA, melanoma S-91, and rat carcinoma 1643, but experimental data were not presented. Extensive studies with podophyllotoxin have also been published regarding its effect on the sarcoma 37 in mice (9).

Clinical experience with podophyllotoxin has been disappointing. In the one available published study, Savel (10) reported that 25 patients with neoplastic disease were studied, of whom 17 were treated to the point of bone marrow or GI toxicity with doses of 0.5–1.0 mg/kg iv. Side effects (nausea, vomiting, diarrhea, oral ulcers, and fever) were frequent. Transient, clinically insignificant regression of tumor masses and lymph nodes occurred in one-third of the patients with reticulum cell sarcoma, two-thirds of the patients with Hodgkin's disease, and one-seventh of the patients with adenocarcinoma. One patient with chronic granulocytic leukemia and rapidly enlarging adenopathy had a marked regression of lymph nodes and spleen size.

α-Peltatin and β-peltatin have been found effective in treating the following types of tumors in laboratory animals: L-1210 leukemia, lymphosarcoma 1, lymphosarcoma 2, mammary adenocarcinoma C3HBA, melanoma S-91, and rat carcinoma 1643 (8), as well as the sarcoma 37 (9), but neither lignan was effective against the Caspari mammary adenocarcinoma (11). However, in clinical trials, α-peltatin by intravenous administration was of no significant therapeutic value in human neoplastic disease (12). β-Peltatin was clinically evaluated in children with dediastinal neuroblastoma with undramatic results (13).

The new lignan, 3′-demethylpodophyllotoxin (III), exhibited an ED_{50} of 1.0 μg/ml against Eagle's 9KB carcinoma and a prolongation of life of 130% T/C at a dose of 1 mg/kg against the P-388 lymphocytic leukemia.

Whereas it does not appear that podophyllotoxin-type lignan aglycones will prove to be effective chemotherapeutic agents for the treatment of human neoplastic diseases, certain derivatives of the water-soluble glycoside forms of these lignans, *i.e.*, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-2-thenylidene)-β-D-glucopyranoside (14–19), have been used clinically with some success.

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