Thermal Chemistry of Podophyllotoxin in Ethanol and a Comparison of the Cytostatic Activity of the Thermolysis Products

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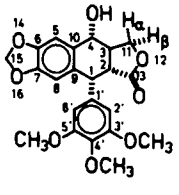
Abstract □ Podophyllotoxin (**1**) in buffered ethanolic solution is degraded by two pathways. One leads to (*a*) picropodophyllin (**2**), which undergoes dehydration to give α-apopicropodophyllin (**5**), which rearranges to give β-apopicropodophyllin (**6**), (*b*) the ethyl ether of picropodophyllotoxin, **8**, and (*c*) the ethyl ether of epipicropodophyllotoxin, **7**. The other pathway leads directly to epipodophyllotoxin (**10**) and the corresponding ethyl ether, **9**, and possibly, via a transient 3,4-dehydropodophyllotoxin (**5'**), to β-apopicropodophyllin (**6**). The ^{1}H NMR spectra of these compounds are described, their in vitro cytostatic activity compared, and their syntheses, including that of podophyllotoxin ethyl ether, reported.

Podophyllotoxin-containing resins from *Podophyllum peltatum* and *P. emodi* have been used medicinally for more than 1000 years. Podophyllin solutions in which the active principle consists of podophyllotoxin and solutions or ointments containing purified podophyllotoxin are the most effective and commonly used medicinal agents for the treatment of venereal warts (*Condyloma acuminata*).[1] Podophyllotoxin itself was determined to be too toxic for systemic administration as a chemotherapeutic agent. This has led to the development of two promising derivatives, etoposide and teniposide.[2,3]

Since podophyllotoxin itself is in clinical use, and since it is the starting material for the synthesis of etoposide and teniposide,[4] we decided to examine its thermal behavior. In the present study, a formulation used in the treatment of venereal warts, 0.5% podophyllotoxin in 96% ethanol buffered with lactic acid:sodium lactate, was examined. Experiments were undertaken at various temperatures ranging from 40 to 180 °C (60–180 °C experiments were conducted in an autoclave) and no qualitative differences in the product distribution were observed.

All of the products have been previously described, but were not acknowledged as thermal degradation products. Also, rigorous structural determinations or syntheses have been reported for only a few of these products. We describe in this report the complete identification of all thermal degradation products and a preliminary in vitro evaluation of their cytostatic activities.



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Results

Chemistry—Ethanolic solutions of podophyllotoxin and picropodophyllin, buffered to pH 4.5 with lactic acid:sodium lactate, were thermally degraded at various temperatures in closed ampules placed in an autoclave containing ethanol in order to have equal pressure inside and outside the ampules. The product distribution was determined by HPLC, and the products were isolated and identified by comparison with authentic samples (IR and HPLC retention times, Fig. 1), except for compounds **10** and **11**, which were only identified by their HPLC retention times.

Podophyllotoxin (**1**) is primarily converted to picropodophyllotoxin (**2**) at each temperature. After ~10% conversion

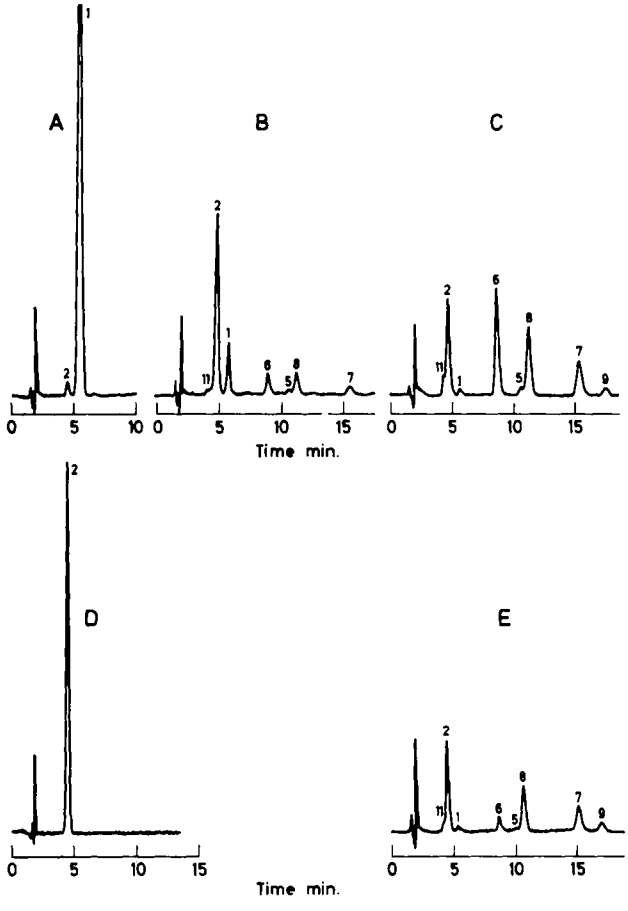

Figure 1—*High-performance liquid chromatography chromatogram of podophyllotoxin* (A, B, *and* C) *and picropodophyllotoxin* (D, E) *degradation at 180 °C taken after:* A, *0 h;* B, *4 h;* C, *16 h;* D, *0 h; and* E, *16 h.*

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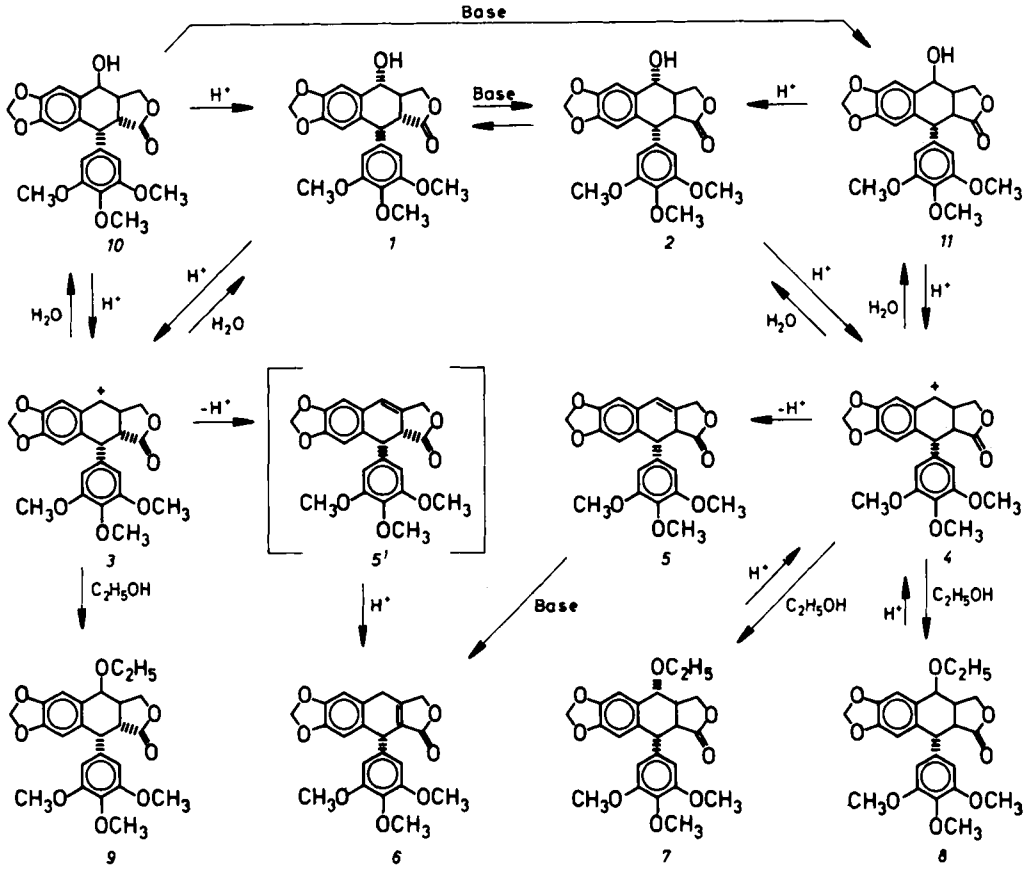

Scheme I

to 2 has taken place, four more products are formed simultaneously, i.e., α-apopicropodophyllin (5), β-apopicropodophyllin (6), picropodophyllotoxin ethyl ether (7), and epipicropodophyllotoxin ethyl ether (8). Upon further thermolysis, epipodophyllotoxin ethyl ether (9) and epipicropodophyllotoxin (11) were observed. Compounds 6, 7, and 8 were generated in a ratio of 3:2:3, whereas compound 5 remained at a low steady-state value. When ≥90% degradation of 1 took place, the predominant product was 6. The product distribution was temperature independent from 40 to 180 °C.

The related lignands, podophyllotoxone or desmethylpodophyllotoxin, were not detected. During thermolysis, the pH of the reaction mixture increased to ~6, which is believed to be due to ethyl lactate formation since the appearance of ethyl lactate was observed by vapor phase chromatography.

The thermolysis of picropodophyllotoxin (2) led to the same pattern as that found after 10% degradation of 1, with the exception that a small amount of 1 was formed.

The 500 MHz ^1H NMR spectra of all of the isolated and synthesized lignans were recorded (Table I), and we have undertaken a total analysis of the spectra with the help of computer simulation (Fig. 2 and Table II). The ^{13}C spectra (Table III) are also in excellent agreement with the assigned structures.

Cytostatic Activity—The cytostatic activity of the podophyllotoxin derivatives was determined by their ability to inhibit the growth of SEWA mouse tumor cells[5] in culture, and the results are presented in Table IV. In accordance with previous reports,[6] picropodophyllotoxin (2) and epipodophyllotoxin (10), as well as the ethyl ether of 10, were much less toxic than podophyllotoxin itself. Most interestingly, however, it was found that the dehydrated forms α-apopicropodophyllin (5) and β-apopicropodophyllin (6), were at least as toxic as podophyllotoxin. Furthermore, alkaline elution analysis (Fig. 3) indicated that these derivatives, in analogy to podophyllotoxin but in contrast to etoposide and teniposide,[7] did not induce protein associated DNA single strand breaks in the cells and that their mode of action thus resembles that of podophyllotoxin.

Discussion

The thermolysis of 1 in buffered ethanol results in products which appear to be generated by two pathways (Scheme I). Podophyllotoxin undergoes C-2-epimerization to 2, which in turn undergoes HO protonation followed by dehydration to give the carbonium ion 4. Simultaneously, but at a slower rate, 1 appears to be converted to its corresponding carbonium ion 3. The carbonium ions are quenched either by deprotonation to give 5, and possibly a transient intermediate such as the C-2 epimer, 5' (which under the experimental conditions, mainly or exclusively rearranges to the more stable 6), or by solvolysis to give three (7–9) of the four possible ethyl ethers, (where 8 can epimerize to 7 by acid catalysis).[5] Quenching with water leads to 10 and 11. Compound 10 easily epimerizes to 11 in the presence of base, while the reverse process takes place under acidic conditions.[8] The formation of a small amount of 1 from 2 is in agreement with the previously reported equilibrium between 1 and 2 under acidic conditions.[8]

The ratio of 7 to 8 (2:3) reflects the expected steric constraints when ethanol attacks the carbonium ion 4. Such an effect should be more pronounced for 3, and this appears to be true since only one of the possible products, i.e., 9 is formed. Likewise, the total lack of formation of podophyllotoxone is also in agreement with related results in the synthesis of glycosides like etoposide and teniposide.[2]

The biological behavior of 2 compared with 1 is also in excellent agreement with previously reported results.[8] The

Table I—Proton NMR Chemical Shifts Assignments for Podophyllotoxin and its Thermal Degradation Products[a]

Compound	1	2	3	4	4″	5	8	11α	11β	15	2′, 6′	OCH_3 (3′, 5′)	$-OCH_3$ (4′)	$-OCH_2CH_3$ (4)	$-OCH_2CH_3$ (4)	OH (4)
Podophyllotoxin (**1**)	4.60	2.84	2.78	4.79		7.22	6.59	4.61	4.09	6.02	6.44	3.76	3.83			2.08
Picropodophyllotoxin (**2**)	4.14	3.23	2.74	4.50		7.07	7.01	4.51	4.44	5.95	6.47	3.83	3.86			2.17
Epipodophyllotoxin (**10**)	4.60	3.27	2.84		4.87	6.88	6.69	4.38	4.35	5.99	6.29	3.75	3.81			2.11
Epipicropodophyllotoxin (**11**)	4.46	3.44	3.10		4.83	7.01	6.61	4.36	4.34	5.97	6.36	3.79	3.83			2.14
α-Apopicropodophyllotoxin (**5**)	4.07	3.72		6.41		6.65	6.24	4.97	4.87	5.92	6.55	3.85	3.90			
β-Apopicropodophyllotoxin (**6**)	4.83			3.65	3.85	6.75	6.66	4.90	4.82	5.96	6.40	3.78	3.79			
Epipodophyllotoxin ethyl ether (**9**)	4.36	3.41	2.87		4.47	6.83	6.54	4.35	4.33	5.91	6.27	3.67	3.81	3.55	1.23	
Picropodophyllotoxin ethyl ether (**7**)	4.09	3.24	2.84	4.14		6.89	6.33	4.42	4.32	5.93	6.46	3.82	3.85	3.68	1.25	
Epipicropodophyllotoxin ethyl ether (**8**)	4.33	3.34	3.12		4.33	6.88	6.48	4.35	4.33	5.93	6.38	3.77	3.83	3.43	1.16	

[a] Reported in parts per million downfield from Me_4Si.

Table II—Proton NMR Coupling Constant Assignments for Podophyllotoxin and its Thermal Degradation Products

Compound	1,2	2,3	3,4″	3,4	4,4″	3,11α	3,11′β	11,11′	2,4	2,11α,β	4,11α,β	1,4″	1,4	1,11β	1,11α
Podophyllotoxin (**1**)	4.5	14.0		9.0		10.0	6.5	9.5							
Picropodophyllotoxin (**2**)	5.0	8.0		8.0		8.5	6.0	9.5							
Epipodophyllotoxin (**10**)	4.5	14.5	3.5			10.3	7.5	8.5							
Epipicropodophyllotoxin (**11**)	4	10.5	5			8.0	4.0	10							
α-Apopicropodophyllotoxin (**5**)	16							0	3	2.5	1.5				
β-Apopicropodophyllotoxin (**6**)					22			17				3	4	2	0
Epipodophyllotoxin ethyl ether (**9**)	5.2	14.0	3.5			11.0	7.8	8.0							
Picropodophyllotoxin ethyl ether (**7**)	5.0	9.7		8.0		6.9	3.0	9.3							
Epipicropodophyllotoxin ethyl ether (**8**)	4.0	11.0	4.5			8.0	3.5	9.0							

Table III—Carbon NMR Chemical Shift Assignments for Podophyllotoxin and its Thermal Degradation Products[a]

Compound	1	2	3	4	5	6	7	8	9	10	11	13	15	1′	2′	3′,5′	4′	6′	OCH_3	OCH_3
Podophyllotoxin (**1**)	44.1	45.2	40.6	71.4	106.4	147.6	147.6	109.6	130.9	133.4	71.4	174.8	101.4	135.6	108.4	152.5	137.0	108.4	56.2	60.8
Picropodophyllotoxin (**2**)	44.0	45.4	42.6	69.3	105.4	147.3	146.9	109.1	130.5	132.0	69.8	177.8	101.1	139.2	105.7	153.5	137.1	105.7	56.2	60.8
Epipodophyllotoxin (**10**)	44.0	40.5	38.4	67.8	109.1	148.5	147.5	110.4	131.9	131.9	66.7	175.3	101.6	135.2	108.3	152.6	137.2	108.3	56.2	60.8
Epipicropodophyllotoxin (**11**)	44.5	45.3	39.5	67.6	106.4	147.4	147.1	109.7	130.1	131.5	68.3	179.2	101.2	137.7	105.0	153.3	136.9	105.0	56.3	60.7
α-Apopicropodophyllotoxin (**5**)	44.5	46.7	120.5	126.9	109.4	147.5	146.6	109.4	131.0	131.4	69.1	174.5	101.2	137.3	107.1	153.5	136.8	107.1	56.2	60.9
β-Apopicropodophyllotoxin (**6**)	42.8	157.4	138.3	29.2	109.5	147.3	147.1	107.8	128.1	123.8	71.0	172.2	101.3	137.2	105.8	153.3	129.6	105.8	56.2	60.8
Epipodophyllotoxin ethyl ether (**9**)	44.0	41.2	38.4	66.0	109.4	148.3	146.8	110.6	129.8	132.1	67.6	175.0	101.4	137.2	108.3	152.5	135.5	108.3	56.3	60.9
Picropodophyllotoxin ethyl ether (**7**)	44.4	45.1	38.4	64.6	107.2	147.5	146.6	109.6	129.7	131.1	68.4	178.7	101.1	137.7	105.4	153.4	137.0	105.4	56.2	60.7
Epipicropodophyllotoxin ethyl ether (**8**)	44.2	45.1	38.4	64.5	107.4	147.5	146.5	109.4	129.7	131.4	68.4	178.8	101.1	137.8	105.6	153.3	136.8	105.6	56.2	60.8

[a] Reported in parts per million downfield from Me_4Si.

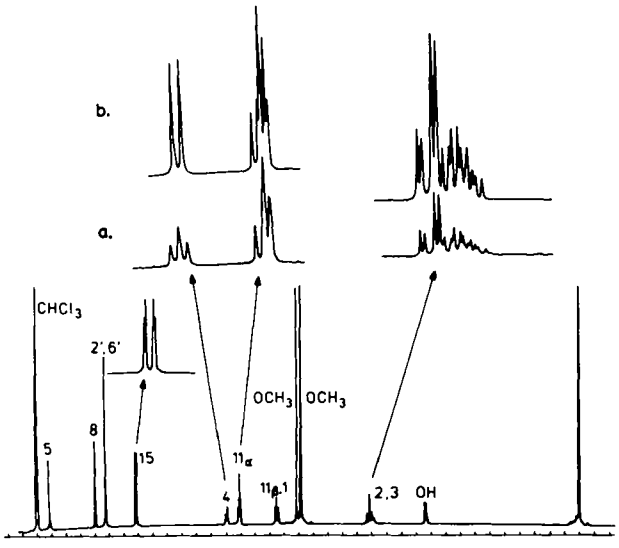

Figure 2—*^{1}H NMR spectrum (500 MHz) of podophyllotoxin (a: enlarged; b: simulated).*

Table IV—Inhibition of the Growth of Mouse Ascites Tumor Cells In Culture

Compound	Relative Cytotoxicity
Podophyllotoxin (**1**)	1
Picropodophyllotoxin (**2**)	0.02
Epipodophyllotoxin (**10**)	0.1
Epipicropodophyllotoxin (**11**)	0.1
α-Apopicropodophyllotoxin (**5**)	1
β-Apopicropodophyllotoxin (**6**)	3
Epipodophyllotoxin ethyl ether (**9**)	0.01
Picropodophyllotoxin ethyl ether (**7**)	0.01
Epipicropodophyllotoxin ethyl ether (**8**)	0.01

ethyl ethers showed only small cytostatic effects, whereas it was surprising to see that β-apopicropodophyllin (**6**) was a more powerful cytostatic agent than podophyllotoxin according to the present assay.

Experimental Section

Materials and Methods—Podophyllotoxin (>98%, HPLC, mp 181–182 °C, $[\alpha]_D^{20}$ −130 ° [lit.[9] 183–184 °C, $[\alpha]_D^{20}$ −132 ° (*c* 1.0, $CHCl_3$)] was provided by pHarma-medica A/S, Copenhagen, Denmark. All other chemicals for synthesis and analysis were commercially available unless described below. Microanalyses are within 0.4% of theoretical values when indicated by symbols of the elements. The ^{1}H NMR spectra were recorded on a Bruker AM 500 spectrometer, and analyzed by the MIMER-1-1984 program. The ^{13}C NMR spectra were recorded on a JEOL FX90Q spectrometer, and EI mass spectra on a AEI MS902 spectrometer at 70 eV. All ^{1}H NMR and ^{13}C NMR spectra were taken in $CDCl_3$ solution with Me_4Si as the internal standard. High-performance liquid chromatography was performed on a Spectra Physics 8000 instrument with a Spherisorb S5 ODS column. The eluant was methanol:potassium dihydrogen phosphate buffer (0.09 M, pH 6.5):acetonitrile (10:9:1), and the flow rate was 1 mL/min. A Microlab gas chromatograph with a 80/100 mesh Gas Chrom Q Column was used to detect changes in the solvent.

Thermolysis—The thermolysis of podophyllotoxin (0.5%) in 96% ethanolic solution buffered with lactic acid:sodium lactate, pH 4.5, (6%:0.8) was carried out in closed ampules (40 mL) and at temperatures >60 °C. These ampules were placed in sealed steel autoclaves containing 96% ethanol. The ampules and the autoclaves were subsequently placed in a thermostated oven at 80, 100, 120, 150 or 180 °C for 4 or 16 h. The samples were all analyzed by HPLC. The samples heated to 40 or 60 °C were analyzed for ethyl lactate by GC, and their pH was determined after thermolysis. In each case ethyl lactate was found. Identical retention times to those of the authentic samples were found.

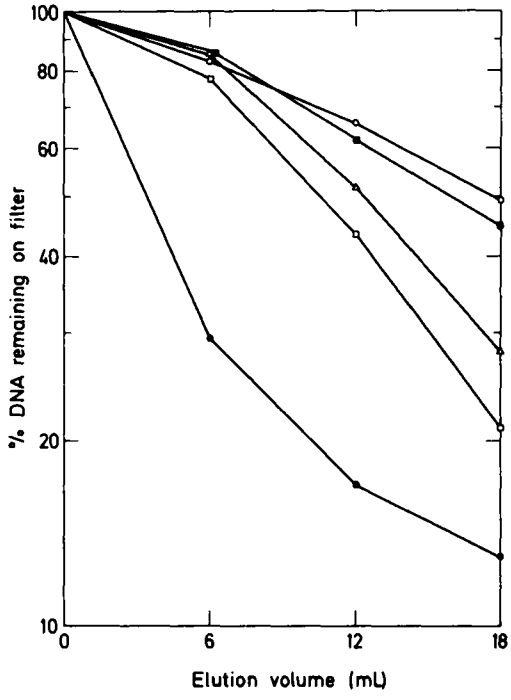

Figure 3—*Alkaline elution analysis of SEWA cells. The cells (10^6 in 1 mL of medium) were incubated for 60 min at 37 °C in the presence of: podophyllotoxin (**1**; 1 μg/mL, △); β-apopicropodophyllin (**6**; 1 μg/mL, □; 0.1 μg/mL, ■); 9-[(2-methoxy-4-methanesulfamido)phenylamino]acridine (mAmsa; 0.1 μg/mL, ●); or without drug (○). The cells were applied on the filters, lysed, treated with protease (0.1 mg/mL, 60 min), and the DNA was eluted at pH 12.1 as previously described (refs 12 and 13).*

Isolation of the Degradation Products—After degradation, the ethanolic solution (200 ml ~1 g podophyllotoxin, 16 h, 180 °C) was filtered to remove the insoluble picropodophyllotoxin (2, 200 mg). The solution was chromatographed on a silica gel $60F_{250}$ column using hexane:ethyl acetate (30:70) as the eluant. The elution gave three fractions, the first of which contained the ethyl ethers of picropodophyllotoxin (7, 120 mg), epipicropodophyllotoxin (8, 140 mg) and epidophyllotoxin (9, 80 mg) which were separated by fractional crystallization. The second fraction contained β-apopicropodophyllin (**6**, 200 mg), and the third fraction contained podophyllytoxin with minor amounts of picropodophyllotoxin, epipicropodophyllotoxin (**11**) and epipodophyllotoxin (**10**). Compounds **2**, **6**, **7**, **8**, **9** were identified by comparison with authentic samples prepared as described below; identical IR and ^{1}H NMR spectra and HPLC retention times, whereas compounds **10** and **11** were only identified by the identity of their retention times with those of the authentic samples. The amount of **10** formed was so small that it could not be found in the standard HPLC analysis even upon 90% degradation.

Synthesis—*Picropodophyllotoxin (2)*—A mixture of 20.0 g of podophyllotoxin, 250 mL of absolute ethanol, and 100 mL of 1 M aqueous sodium acetate was refluxed and stirred for 12 h. The mixture was cooled to 4 °C and filtered. Washing with ethyl acetate and recrystallization from absolute ethanol gave 15.6 g (78%) of picropodophyllotoxin: $[\alpha]_D^{20}$ +4.5 ° (*c* 1, $CHCl_3$); mp 229–231 °C (lit.[9] $[\alpha]_D^{20}$ +4.8 °, mp 227–230 °C); IR: 1765, 1590, and 1480 cm^{-1}; $[M^+]$ 415. Anal. ($C_{22}H_{22}O_8$) C, H.

β-Apopicropodophyllin (6)—A mixture of 2.0 g of α-apopicropodophyllin, 20 mL of acetic acid, and 2 mL of piperidine was refluxed for 1 h, after which time it was cooled and diluted with 200 mL of water. After 3 h in the refrigerator, colorless needles separated. Recrystallization from acetic acid gave 1.4 g (70%) of β-apopicropodophyllin: $[\alpha]_D^{20}$ +95.8 ° (*c* 1, $CHCl_3$); mp 216–217 °C [lit.[10] +97 ° (*c*, 0.5, $CHCl_3$); mp 220–222 °C]; IR: 1755, 1590, and 1485 cm^{-1}; $[M^+]$ 396. Anal. ($C_{22}H_{20}O_7$) C, H.

Podophyllotoxin Bromide—A solution of 20.2 g of anhydrous podophyllotoxin in 200 mL of benzene was refluxed with 4.6 g of phosphorous bromide for 1 h. The supernatant was decanted and the residue was washed with 2 × 50 mL of hot benzene. The benzene was

removed under reduced pressure (30 °C, 15 mmHg). The resulting glassy material was taken up in 100 mL of warm benzene, which again was removed under reduced pressure at 30 °C. The last traces of benzene were removed by further heating at 70 °C for 1.5 h under reduced pressure to give 22.4 g (96%) of a white foam which was used without further purification.

Epipodophyllotoxin (10)—Epipodophyllotoxin bromide (5.0 g), 40 mL of acetone, and 40 mL of water in the presence of 10 g of powdered $BaCO_3$ was refluxed for 1 h. Epipodophyllotoxin was removed from the $BaCO_3$ by extraction with 100 mL of boiling acetone. After filtration the solvent was removed by evaporation at room temperature and 15 mmHg. This extraction/filtration/evaporation procedure was repeated twice. The last traces of solvent were removed by further heating at 76 °C under reduced pressure for 2 h. After drying under reduced pressure (1 mmHg) at 110 °C, a semisolid product, 4.4 g (93%), was obtained. Two recrystallizations from 50% ethanol gave analytically pure material that showed one peak on HPLC. $[\alpha]_D^{20}$ −74.5° (*c* 1, $CHCl_3$); mp 161 °C (lit.[9] $[\alpha]_D^{20}$ −75°, mp 159–161 °C); IR: 1770, 1590, and 1485 cm^{-1}; $[M^+]$ 414. Anal. ($C_{22}H_{22}O_8$) C, H.

α-Apopicropodophyllin (5)—Ion exchange resin (Amberlyst, 2.0 g) was added to a solution of 2.0 g of picropodophyllotoxin in 12 mL of boiling acetic anhydride. After 30 min, the solution was filtered and kept in the refrigerator overnight. Colorless needles separated which were removed by filtration and recrystallized two times from acetic acid containing traces of HCl, to give 1.6 g (84%) of α-apopicropodophyllin: $[\alpha]_D^{20}$ −11.6° (*c* 1, $CHCl_3$); mp 237 °C (lit.[11] $[\alpha]_{546}^{21}$ −17.5°, mp 236–237 °C); IR: 1780, 1590, and 1480 cm^{-1}; $[M^+]$ 396. Anal. ($C_{22}H_{20}O_7$) H, C: calcd, 66.66; found, 65.97.

Epipicropodophyllotoxin (11)—Epipodophyllotoxin (3.2 g) in 200 mL of 75% aqueous ethanol was refluxed in the presence of 0.06 mL of piperidine for 8 h. After this time the solvent was removed at 30 °C under reduced pressure. Residual traces of water were removed by dissolution of the solid in absolute ethanol and removal of the solvent at 30 °C under reduced pressure, then drying for 2.5 h at 75 °C under reduced pressure. This gave 3.0 g (95%) of a semicrystalline material which was recrystallized from methanol: $[\alpha]_D^{20}$ +75.4° (*c* 1, $CHCl_3$); mp 159 °C (lit.[9] $[\alpha]_D^{20}$ +85°, mp 158–159 °C); IR: 1760, 1590, and 1480 cm^{-1}; $[M^+]$ 414. Anal. ($C_{22}H_{22}O_8$) C, H.

Picropodophyllotoxin Ethyl Ether (7) and Epipicropodophyllotoxin Ethyl Ether (8)—To a solution of 4.0 g of picropodophyllotoxin in 400 mL of absolute ethanol was added 4.0 g of ion exchange resin (Amberlyst) and the mixture was refluxed with stirring for 2 h, after which time HPLC showed no peak for the starting material. The ion exchange resin (Amberlyst) was removed by filtration and the remaining solution was cooled at 4 °C for 20 h. The precipitated material, which consisted of epipicropodophyllotoxin ethyl ether (8, 1.3 g) was isolated. By reducing the volume of the solution to ~200 mL and cooling, a further crop of 8 (1.0 g,) was precipitated and isolated. The total yield of 8 was 52%. The remaining solution was evaporated to dryness to give 1.5 g (34%) of picropodophyllotoxin ethyl ether (7).

Epipicropodophyllotoxin ethyl ether (8): $[\alpha]_D^{20}$ +45.7° (*c* 1, $CHCl_3$); mp 143 °C (lit.[9] $[\alpha]_D^{20}$ +45°, mp 151–153 °C); $[M^+]$ 442; IR: 1765, 1590, and 1485 cm^{-1}. Anal. ($C_{24}H_{26}O_8$) C, H.

Picropodophyllotoxin ethyl ether (7): $[\alpha]_D^{20}$ +74.9° (*c* 1, $CHCl_3$); mp 198 °C (lit.[7] $[\alpha]_D^{21}$ +58° (*c* 0.5, $CHCl_3$), mp 227–229 °C); IR: 1775, 1590, and 1480 cm^{-1}; $[M^+]$ 442. Anal. ($C_{24}H_{26}O_8$) C, H.

Epipodophyllotoxin Ethyl Ether (9)—A suspension of 4.8 g of freshly prepared podophyllotoxin bromide and 10 g of powdered barium carbonate in 80 mL of absolute ethanol was stirred under reflux for 1 h. The mixture was then concentrated under reduced pressure. The solid material was extracted with acetone as described above for epipodophyllotoxin (**10**). Several recrystallizations from 50% ethanol, followed by drying under reduced pressure (0.1 mmHg, 100–110 °C), gave 4.1 g (80 %)of analytically pure epipodophyllotoxin ethyl ether (**9**): $[\alpha]_D^{20}$ −87.4° (*c* 1, $CHCl_3$); mp 192 °C (lit.[9] $[\alpha]_D^{20}$ −88°, mp 194–198 °C); $[M^+]$ 442; IR: 1775, 1590, and 1485 cm^{-1}. Anal. ($C_{24}H_{26}O_8$) C, H.

Cytotoxicity—SEWA mouse tumor cells were maintained in culture at 37 °C in McCoy's medium (Gibco) containing 10% heat-inactivated fetal calf serum. For toxicity assays, the cells were seeded in microtiter wells (200 μL) at 5×10^3 cells/mL. Ten-fold (and also three-fold for compounds **5**, **6**, **10**, and **11**) dilutions of the compounds ranging from 10 μg/mL to 1 ng/mL were tested at least in duplicate, and the concentrations causing total inhibition of cell growth (ID_{100}) after 6 d were determined by visual inspection of the plates (high cell density decreases the pH of the medium which changes the color from red to yellow). The visual scoring was confirmed by light-microscopic inspection of the wells. Podophyllotoxin was included in each test series as a standard and consistently showed toxicity (ID_{100}) at ~10 ng/mL.

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