Synthesis and Biological Evaluation of New Selective Cytotoxic Cyclolignans Derived from Podophyllotoxin†

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Podophyllotoxin and some of its derivatives are cyclolignans currently used for removing warts and in the clinical treatment of malign neoplasms. As such, they have been an objective of the scientific community for decades, in the search for more potent and more selective anticancer agents. Our interest in the chemoinduction of drug selectivity led us to the design and preparation of new podophyllotoxin derivatives by reaction of podophyllic aldehyde with aliphatic, aromatic, and heteroaromatic amines. Several of the resulting imines displayed a significant selectivity against human colon carcinoma cells, even higher than that of the starting aldehyde. Additional biological studies indicate that these derivatives induce microtubule depolymerization, arrest cells at the G_2/M phase of cell cycle, and are able to induce a delayed apoptosis after 48 h of treatment, characterized by caspase-3 activation.

Introduction

Cyclolignans are a large family of natural products widely distributed in the plant kingdom.[1] They display a variety of biological activities, which have attracted the attention of the scientific community for decades.[2] Particularly interesting is podophyllotoxin **1**, the main component of *Podophyllum* resin, whose medicinal properties have been well-recognized for centuries.[3] Podophyllotoxin has been used as cathartic, antirheumatic, antiviral, etc., but its antitumor activity has proved to be the most attractive, making this cyclolignan a lead compound for drug design in the search for improved pharmacological profiles.

Thus, several hundred derivatives were prepared, culminating with the clinical introduction of some semisynthetic analogues named etoposide[4] and teniposide,[5] and more recently etopophos,[6] a more soluble prodrug of etoposide (Figure 1). Surprisingly, these semisynthetic derivatives and the parent compound, podophyllotoxin, showed different mechanisms of action.[2] Podophyllotoxin inhibits the assembly of tubulin into microtubules through interaction with the protein at the colchicine binding site, preventing the formation of the spindle. The result is that the cells, which have begun to divide, are arrested in the metaphase (G_2/M stage), remaining with their chromosomes joined together until they disintegrate several hours later.

Etoposide and congeners are not inhibitors of microtubule formation.[2] They induce a premitotic blockade

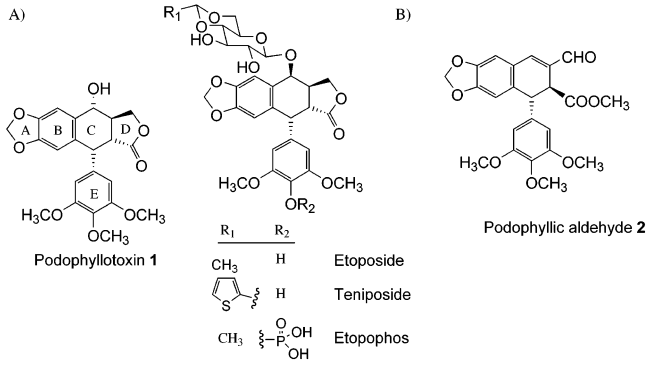

Figure 1. Structures of podophyllotoxin and related compounds. (A) Cyclolignans in clinical use. (B) Selective cytotoxic aldehyde.

in late S stage of the cell cycle because of the inhibition of DNA topoisomerase II (Topo II), an enzyme required for the unwinding of DNA during replication. Etoposide binds to and stabilizes the DNA−protein complex preventing religation of the double-stranded breaks.

The chemical changes that transform podophyllotoxin from a compound that interacts with tubulin into a molecule that inhibits Topo II are demethylation at C-4′, epimerization at C-7, glucosylation at C-7, and acetalization of the 4- and 6-hydroxyl groups of the glucopyranose units using aldehydes.

These are two well-known mechanisms of action for podophyllotoxin derivatives, but there must be at least one additional unknown mechanism, because some derivatives have been reported to be as cytotoxic as podophyllotoxin and etoposide, but do not inhibit tubulin polymerization and are only very weak inhibitors of Topo II in vitro.[7] On the other hand, it is necessary to bear in mind the ability of these anticancer agents to induce apoptosis. It has been reported that both anti-

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microtubule agents (such as podophyllotoxin[8] and deoxypodophyllotoxin[9]) and Topo II inhibitors (such as etoposide[10] or GL331[11]) induce apoptotic cell death through independent mechanisms and that would also contribute to their cytotoxicity.

These derivatives are widely used as anticancer drugs, but they still have several secondary effects. Because of this, the aryltetralin lignans are still the subject of extensive research. Considering the cyclolignan skeleton, nearly every ring of the molecule (A to E) has been modified.[12] Our group has been involved for several years in the chemical transformation of podophyllotoxin and its derivatives and has prepared a large number of cyclolignans by modifications of nearly all the rings of the skeleton,[12b,13−15] looking for more potent, less toxic and, if possible, more selective analogues. Our group has proposed that the main point of interaction between cyclolignanolides and biomacromolecules is centered at C-9[16] rather than at C-9′ as previously proposed by Eich,[17] and this proposal has been confirmed chemically through the observation that weak nucleophiles are able to open the lactone ring by attack at position C-9.[16,18] The ease with which the reaction occurs at C-9 prompted us to modify the electrophilic character at that position, and the corresponding podophyllic aldehyde **2** (Figure 1) was prepared and evaluated. This aldehyde not only retained the cytotoxicity but also showed an interesting selectivity against the HT-29 colon carcinoma[19] and thus became our lead compound for further transformations.

We had obtained a cytotoxic and selective cyclolignan lacking the γ-lactone ring, generally considered an important feature for the bioactivity of podophyllotoxin analogues.[2,12] This is not the only case in which modifications of a lactone ring—considered essential for the interaction with the target enzymes—led to improved pharmacological profiles; an example can be found on camptothecin analogues modified in the lactone ring which retained the cytotoxicity.[20] In this sense several of the modifications reported in this paper could also be appropriate for the camptothecin too.

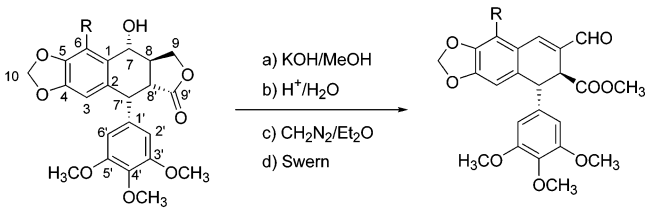
Following this research, we now report the synthesis of a number of imine derivatives that had not only maintained the cytotoxicity of the parent, but have also considerably improved the selectivity against the HT-29 colon carcinoma. Also, additional biological studies have been performed in order to analyze how these cyclolignans affect the cell cycle.

Chemistry

The natural products podophyllotoxin **1** and 6-methoxypodophyllotoxin **3** were transformed into the podophyllic aldehydes **2** and **4**, and both aldehydes were the starting materials for the transformations presented here. Podophyllotoxin **1** was isolated from *Podophyllum* resin,[14] and **3** was kindly supplied by Prof. N. Prass (University of Groningen) as its β-D-glucoside.[21] The numbering of compounds used in this paper is in accordance with the accepted IUPAC numbering of lignans.[22]

Cyclolignans **1** and **3** were transformed into the aldehydes **2** and **4** (Scheme 1) through opening of the lactone ring under basic conditions, followed by methylation of the carboxylic acid and oxidation of the resulting dihydroxy-ester under Swern conditions, as previously reported by us.[19]

Scheme 1. Preparation of Podophyllic Aldehydes **2** and **4**



R	Compound	Compound
H	**1**	**2**
OCH_3	**3**	**4**

The imine derivatives were prepared by condensation of the aldehydes with aliphatic, aromatic, and heteroaromatic amines as shown in Scheme 2. It is well-known that aldimines can be very reactive, unstable, and very sensitive to hydrolysis, factors that make both following the reaction and purification of the products difficult. To overcome some of these problems, first it is necessary to bring the reaction to completion by shifting the imine formation equilibrium[23] by removal of water and/or by using excess of the corresponding amine.

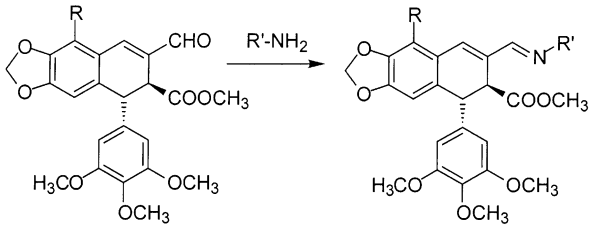
The removal of water was achieved using drying agents[24] such as magnesium sulfate or molecular sieve (methods A−C, see Experimental Section) or azeotropically by distillation[25] using a Dean−Stark apparatus (method D). Monitoring the reaction by TLC was not sufficient to determine whether the reaction was complete, since the hydrolysis products were always observed on the TLC. Thus, the reactions were followed by the ^{1}H NMR spectra of aliquots until the signal due to the aldehyde proton disappeared completely.

Using the amines in excess made the purification of the corresponding imines difficult, and only the aryl/heteroaryl imines were stable enough to allow chromatography on silica gel (compounds **13**−**23**); in some cases the silica was deactivated with 1% Et_3N. The aliphatic imines decomposed on attempted column chromatography, and only the precursor aldehyde was recovered. In these cases, the imine was separated from the excess of amine by precipitation from *n*-hexane (derivatives **8** and **9**) or by removal of the amine under vacuum for sufficiently volatile amines, such as was the case for **5**−**7** and **10**−**12**.

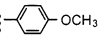
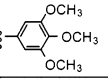
Spectroscopic data of the final products confirm the quantitative transformation of the aldehyde into the imines. The structures were also confirmed by heteronuclear 2D-NMR correlations (HMBC, HMQC) for some of the imines prepared. The configuration of the imine double bond was determined by NOE experiments: positive NOE's were observed in the signals of H-7 and H-1″ when the H-9 signal was irradiated, confirming the anti configuration of the imine.

Under these conditions, the reaction time was long (1−30 days). A solvent-free method under microwave irradiation was used in an attempt to reduce reaction time, using calcium carbonate and montmorillonite K 10 clay[26] as solid supports. In these cases, the reaction times were considerably reduced (to a few minutes), but the extraction from the solid support decreased the

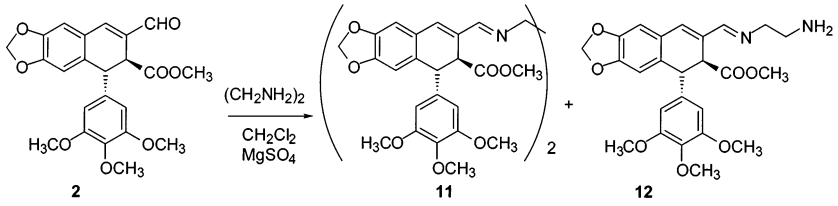
Scheme 2. Preparation of Imine Derivatives of Podophyllic Aldehydes



2, 4 **5-23**

Compound	R	R'	Compound	R	R'
5	H	$C^{(1'')}H_2$-$C^{(2'')}H_3$	**16**	H	
6	OCH_3	CH_2-CH_3	**17**	H	
7	H	CH_2-CH_2-CH_3	**18**	H	
8	H	$(CH_2)_5$-CH_3	**19**	H	
9	H		**20**	H	
10	H	CH_2-CH_2OH	**21**	H	
13	H		**22**	H	
14	H		**23**	H	
15	H				

Scheme 3. Reaction with **2** with Ethylenediamine



2 **11** **12**

Equiv. $(CH_2NH_2)_2$	Reaction time	**11** %	**12** %
3	20 h	100	-
10	75 min	6	94
75	40 min	-	100

yields and a small percentage of aldehyde was always recovered, as shown in the ^{1}H NMR spectra of the reaction products.

When the amine was ethylenediamine, both nitrogen atoms could react with the aldehyde function and, depending on the ratio amine/aldehyde, the diimine **11** or the monoimine **12** was isolated, as summarized in Scheme 3.

Biological Results and Discussion

Cytotoxic Evaluation. Most of the cyclolignans reported here were evaluated in vitro for their cytotoxicities[27] against the following tumor cell lines: P-388 (lymphoid neoplasma from DBA/2 mouse), A-549 (human lung carcinoma), HT-29 (human colon carcinoma), and MEL-28 (human melanoma). The results obtained are shown in Table 1.

All the compounds tested are derivatives of the podophyllic aldehyde **2** and, as can be observed in Table 1, all of them maintained the main biological property: the cytotoxic selectivity against the HT-29 cell line, improved in some cases compared to that of compound **2**. The best IC_{50} values found are in the nanomolar range, even below those of podophyllotoxin (**5**, **14** vs **1**) and other cyclolignanolides, despite the lack of the γ-lactone ring, generally considered an important structural feature for the cytotoxicity of this type of compound.

All the imines tested showed IC_{50} values below the micromolar level, without great differences among them. However, some differences can be observed in the selectivity index (SI) included in Table 1. With the aim of quantifying the selectivity, we compare the values of IC_{50} obtained for these compounds against the cell lines

Table 1. Antineoplastic Activity of Cyclolignan Derivatives (IC_{50}, μM)

compd	P-388	A-549	HT-29	MEL-28	[SI][a]
1	0.012	0.012	0.024	–	0.5
2	0.23	0.12	0.012	0.23	19
4	1.1	0.22	0.020	–	55
5	1.1	0.022	0.0022	0.022	**500**
6	–	>10	0.10	1.0	–
7	1.1	0.11	0.010	–	**110**
8	2.0	0.20	0.020	0.49	**100**
9	2.0	0.25	0.025	0.49	80
10	0.21	0.11	0.021	0.21	10
11	–	0.11	0.034	–	–
13	0.50	0.020	0.020	1.2	25
14	0.94	0.094	0.0094	–	**100**
15	0.85	0.21	0.021	0.42	40
16	–	>9.7	0.97	>9.7	–
19	0.90	0.90	0.20	0.90	4.5
20	1.9	0.023	0.023	1.0	83
21	0.96	0.96	0.048	0.96	20
22	0.20	0.25	0.025	0.25	8

[a] [SI]: Selectivity Index. Ratio IC_{50}(P-388)/ IC_{50}(HT-29).

HT-29 and P-388. Podophyllotoxin was twice as potent against P-388 as against HT-29, and thus has a selectivity factor of 0.5, i.e., it is not selective against HT-29. The aldehyde **2** is about 20 times more active against the HT-29 cell line than against the P-388 cell line; hence, the SI factor observed in this case is 20. Among all the imines obtained, the ethylimine **5** shows the largest selectivity index, being 500 times more potent against HT-29 than against P-388 cells. With this compound the selectivity increased by a factor of 25 compared to that observed for the lead compound **2**.

In general, the aliphatic imines were more selective than the aromatic and heterocyclic analogues, though with some exceptions. The hydroxyethylimine **10** showed a SI of 10, although the potency against HT-29 was similar to that of the rest of the imines, whereas **14** and **20** were less potent against P-388, and this increased the SI to 100 in these cases.

As discussed above, the aliphatic imines were less stable than the aromatic ones, and it is possible, a priori, that the cytotoxicities of these analogues could be due to their capacity to release the aldehyde **2** during the assay. However, the fact that the SI was considerably increased in some cases cannot be explained if only the aldehyde were responsible for the activity, suggesting that the imine moiety plays an important role in the overall activity profile of these compounds. In this sense, further investigation would be necessary into other factors, such as modifications in the cellular uptake or metabolism during migration from the cellular membrane to the nucleus that may contribute to the cytotoxicity of these cyclolignans.

The podophyllic aldehyde **2** was submitted to the NCI evaluation on its 60-tumor cell line panel.[28] Again, selectivity was observed for colon carcinoma cell lines (log TGI(M) = −7.0) and also for CNS and breast cancers (log TGI = −6.1 and −6.3, respectively), while the compound appeared nearly inactive against the rest of the cell lines tested (log TGI = −4.0). The compound did not appear to be a strong MDR substrate since the same potency was measured in strong-MDR and non-MDR cell lines (log TGI = −4.0).

Dose−Response and Time-Course Effects of Cyclolignans on Cell Cycle and Apoptosis in Human Tumor Cells. It has been previously reported that aldehyde **2** disrupts the microtubule cytoskeleton by direct interaction with tubulin.[29] On these grounds we studied the action on the cell cycle of the lead compound **2** and the imines **14** and **21**, as well as the starting material podophyllotoxin **1**, by flow cytometry. Aldehyde **2** was a very potent inhibitor of cell proliferation using the distinct tumor cell lines shown in Table 1. It was also very active in inhibiting proliferation of additional human tumor cell lines, including the human epitheloid cervix adenocarcinoma HeLa cells ($IC_{50} = 0.015 \pm 0.005$ μM, $n = 3$). Cell cycle analysis[30] showed that cells were arrested at the G_2/M phase following treatment with **1**, **2**, **14**, and **21** (Figure 2 and Table 2), which is in agreement with tubulin being a target for these compounds. This cell cycle arrest was dose- and time-dependent with a relative potency of the assayed compounds being **1** > **2** > **14** > **21** (Figure 2 and Table 2). Higher concentrations and longer incubation times are required for the less potent analogues in order to get a similar cell cycle response to that achieved by the most potent compounds (Figure 2 and Table 2). Incubation of HeLa cells with 10 nM to 1 μM aldehyde **2** resulted in accumulation of cells in G_2/M in a time-dependent manner (Figure 2 and Table 2). Similar results were obtained with human colon adenocarcinoma HT-29 cells (data not shown). The concentrations required to elicit these effects on cell cycle are in agreement with the corresponding IC_{50} values measured in the previous cytotoxic assays, which mainly detect inhibition in cell proliferation, because the number of cells used for flow cytometry analysis exceeded in about 25−50 times those figures used in cytotoxic tests. Over 80% of the HeLa cells were arrested at G_2/M with 4n content of DNA after 24-hour incubation with either 100 nM or 1 μM of **2** (Figure 2 and Table 2). This G_2/M arrest led eventually to cell death, and about 30% apoptosis was observed after 48 h of treatment of HeLa cells with 1 μM of **2** (Table 2), as assessed by the appearance of cells with a DNA content less than G_1 (sub-G_1), a characteristic of apoptotic cells. This rather delayed

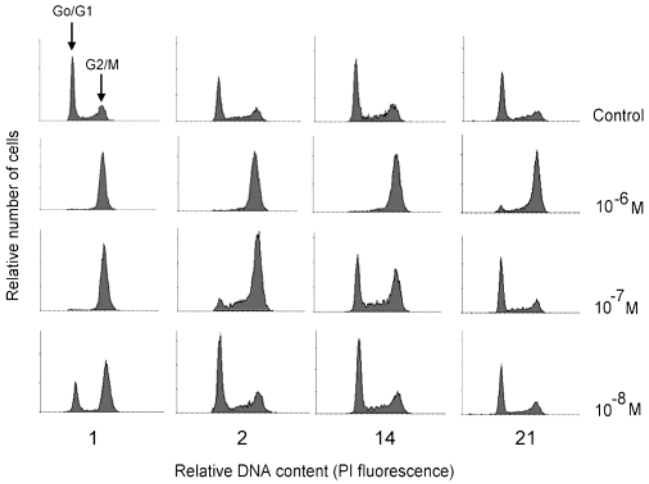


Figure 2. Dose−response of the effects of compounds **1**, **2**, **14**, and **21** on cell cycle in HeLa cells. Cells were incubated with different concentrations of the indicated compounds for 24 h, and their DNA content was analyzed by fluorescence flow cytometry. The position of the G_0/G_1 and G_2/M peaks are indicated by arrows. The experiment was repeated four times and representative histograms are shown.

Table 2. Effect of **1**, **2**, **14**, and **21** on Cell Cycle and Apoptosis in HeLa Cells[a]

treatment		% cells											
		24 h				48 h				72 h			
compd	dose (M)	Sub-G_1	G_0/G_1	S	G_2/M	Sub-G_1	G_0/G_1	S	G_2/M	Sub-G_1	G_0/G_1	S	G_2/M
control		1.2	57.4	17.5	23.9	1.7	57.2	16.3	24.8	2.3	59.0	17.6	21.1
1	10^{-6}	1.5	2.2	4.2	92.1	62.3	9.7	10.0	18.0	89.3	7.1	3.4	0.2
	10^{-7}	1.6	2.2	4.5	91.7	59.0	11.0	10.5	19.5	88.1	9.2	2.5	0.2
	10^{-8}	1.4	22.6	4.3	71.7	68.4	16.1	9.7	5.8	81.8	13.9	4.1	0.2
2	10^{-6}	1.1	1.2	1.7	96.0	28.9	17.0	9.3	44.8	58.9	14.1	17.5	9.5
	10^{-7}	1.4	8.1	10.4	80.1	10.9	11.2	9.7	68.2	50.1	15.1	17.3	17.5
	10^{-8}	1.4	58.1	16.2	24.3	1.5	58.7	10.1	29.7	3.1	47.4	10.2	39.3
14	10^{-6}	1.9	2.0	5.3	90.8	25.3	20.9	6.0	47.8	49.4	19.4	20.8	10.4
	10^{-7}	1.6	33.5	18.8	46.1	5.2	7.7	6.2	80.9	27.1	35.1	13.6	24.2
	10^{-8}	1.4	55.1	15.3	28.2	1.9	57.5	15.1	25.5	2.7	59.3	11.4	26.6
21	10^{-6}	2.1	6.8	9.6	81.5	23.8	16.0	10.8	49.4	39.6	31.3	8.1	21.0
	10^{-7}	1.8	55.6	18.3	24.3	1.9	49.5	13.0	35.6	5.6	37.7	12.3	44.4
	10^{-8}	1.3	56.4	18.2	24.1	1.7	56.0	14.1	28.2	2.3	60.1	12.2	25.4

[a] HeLa cells were incubated with the above compounds in the concentration range of 10^{-6} to 10^{-8} M for the indicated times, and the proportion of cells in each phase of the cell cycle was quantitated by flow cytometry. Cells in the sub-G_1 region represent apoptotic cells. Untreated control cells were run in parallel. Data shown are representative of three independent experiments performed.

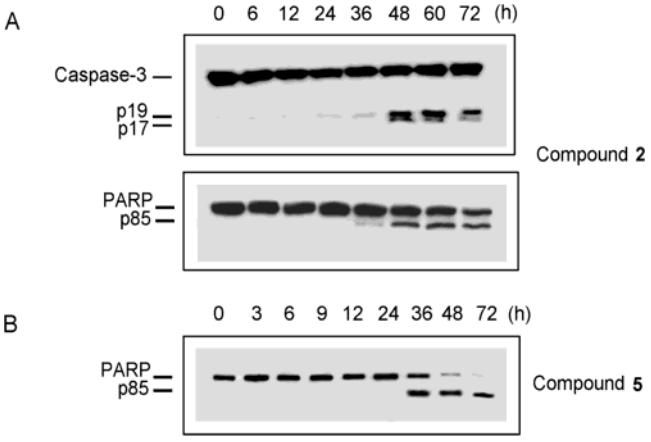


Figure 3. Time-course of caspase-3 and PARP cleavage in HeLa cells following treatment with compounds **2** and **5**. Cells were treated with 1 μM aldehyde **2** (A) or 1 μM compound **5** (B) for the indicated times and analyzed by immunoblotting with anti-caspase-3 and anti-PARP antibodies. The migration positions of full-length caspase-3 and the p19 and p17 cleavage products, as well as of full length PARP and its cleavage product p85 are indicated. Data shown are representative of three experiments performed.

apoptotic response induced by **2** was biochemically characterized by the activation of caspase-3.[31] We found that aldehyde **2** induced caspase-3 activation in HeLa cells as assessed both by cleavage of procaspase-3 into the p17 active form, and by cleavage of the typical caspase-3 substrate PARP (Figure 3). These markers were detected using a polyclonal anti-human caspase-3 antibody that recognized the 32-kDa proenzyme (procaspase-3) and the 17-kDa form of the active caspase-3, and the anti-PARP C2.10 monoclonal antibody that detected both the 116-kDa intact form and the 85-kDa cleaved form of PARP (Figure 3). The caspase-3 cleavage product p19 observed in Figure 3A represents the p17 subunit plus the short caspase-3 prodomain, which is then slowly converted into the active p17 subunit.[32] Activation of caspase-3 was detected by 36 h treatment of HeLa cells with 1 μM aldehyde **2**, and was very evident after 48 h treatment (Figure 3). Incubation with the aspartate-based caspase inhibitor z-Asp-2,6-dichlorobenzoyloxymethyl ketone (50 μM),[30,31] a general caspase inhibitor, blocked the apoptotic response induced by **2** (28% apoptosis vs 4% apoptosis in HeLa cells treated with **2** for 48 h in the absence or presence of the caspase inhibitor respectively). This further suggests the involvement of caspase-3 in aldehyde **2**-mediated apoptosis. Furthermore, fluorescence confocal microscopy analysis showed that treatment of HeLa cells with 1 μM aldehyde **2** led to a rapid (less than 3 h incubation) and extensive microtubule depolymerization (data not shown), but apoptosis was detected by 48 h incubation (Table 2). This indicates the existence of a rather long lag between microtubule disassembly and the onset of apoptosis.

Because the ethylimine **5** showed a high cytotoxic activity against a number of tumor cell lines (Table 1), we analyzed its effect on both cell cycle and caspase-3 activation in comparison to the podophyllic aldehyde **2**. Compound **5** (1 μM) arrested HeLa cells completely (over 98%) at G_2/M phase after 24 h treatment, and induced a slightly more potent apoptotic response than compound **2** after 48 h incubation (44.2% vs 28.9% apoptosis in cells treated with 1 μM **5** or 1 μM **2**, respectively). This **5**-induced apoptotic response was accompanied by caspase-3 activation, assessed by PARP degradation, that was conspicuous following 36 h incubation (Figure 3B). The level of PARP degradation was higher in **5**-treated cells as compared to **2**-treated cells (Figure 3). These effects on caspase-3 activation and apoptosis could explain the higher cytotoxic capacity elicited by the ethylimine **5** in distinct tumor cell lines (Table 1).

Experimental Section

Chemistry. NMR spectra were recorded on a Bruker AC 200 at 200 MHz for ^{1}H and 50.3 MHz for ^{13}C in deuteriochloroform with TMS as an internal standard. Chemical shift values are expressed in ppm followed by multiplicity and coupling constants (*J*) in Hz. The complete NMR signals are given for the first imine described, and only characteristic signals are indicated for the remainder. 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 Nicolet Impact 410 spectrophotometer and wavenumbers are given in cm^{-1}. HRMS were run in a VG-TS-250 spectrometer working at 70 eV. Elemental analyses (C, H, N) were obtained with a LECO CHNS-932 and were within ±0.4% of the theoretical values.

Isolation and Preparation of Compounds 1−4. Podophyllotoxin **1** was isolated from *Podophyllum emodi* resin,[14]

and 6-methoxypodophyllotoxin **3** was obtained by hydrolysis of 6-methoxypodophyllotoxin β-D-glucoside kindly provided by Prof. N. Pras (Groningen, The Netherlands).[21] Both were transformed into the aldehyde derivatives methyl 9-deoxy-9-oxo-α-apopicropodophyllate **2**, and methyl 9-deoxy-6-methoxy-9-oxo-α-apopicropodophyllate **4** by previously described methods.[19]

General Methods for the Synthesis of Imines 5−23. Method A: A mixture of aldehyde (**2** or **4**) (0.10−0.25 mmol), anhydrous $MgSO_4$ (2.0 equiv), and the corresponding amine (1.0 equiv) was dissolved/suspended in CH_2Cl_2 (3.0 mL) and stirred at room temperature during 1−30 d. The reaction mixture was filtered, and the filtrate was concentrated under vacuum to give the imines in quantitative yields unless otherwise specified.

Method B: A mixture of aldehyde **2** (0.10−0.25 mmol), molecular sieves (1:1 w/w with the aldehyde), and the corresponding amine (0.95 equiv) was dissolved/suspended in CH_2Cl_2 or C_6H_6 (15 mL) and stirred at room temperature during 7−14 d. The reaction mixture was filtered off and the filtrate concentrated under vacuum. Residual aldehyde was eliminated after precipitation in *n*-hexane, the corresponding imine being obtained in over 90% yield.

Method C: A mixture of aldehyde **2** (0.10−0.25 mmol), anhydrous $MgSO_4$ (2.0 equiv), and the corresponding amine (1.0 equiv) was dissolved/suspended in dry EtOH (3.0 mL), stirred, and heated under reflux during 1−4 d. After filtering off the reaction mixture, the solvent was removed under vacuum to give the corresponding imines in quantitative yields unless otherwise specified.

Method D: The aldehyde **2** (0.10−0.25 mmol) and the corresponding amine (1.5−10 equiv) were dissolved in C_6H_6 (10−25 mL), stirred, and heated under reflux with a Dean−Stark collector during 2−4 d. The solvent was removed under vacuum, and the reaction product was chromatographed to yield the corresponding imine.

9-Ethylimine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (5). From aldehyde **2** (139 mg, 0.326 mmol), anhydrous $MgSO_4$ (397 mg, 3.27 mmol), and ethylamine 70% in water (1.07 mL, 13.2 mmol) using method A during 3 d. ^{1}H NMR ($CDCl_3$): δ 1.16 (t, 3H, J = 7.1 Hz, NCH_2CH_3), 3.49 (m, 2H, NCH_2CH_3), 3.61 (s, 3H, C9′-OCH_3), 3.73 (s, 6H, C3′-OCH_3, C5′-OCH_3), 3.78(s, 3H, C4′-OCH_3), 4.23 (d, 1H, J = 3.3 Hz, H8′), 4.52 (d, 1H, J = 3.3 Hz, H7′), 5.94 (d, 1H, J = 1.3 Hz, H10a), 5.95 (d, 1H, J = 1.3 Hz, H10b), 6.30 (s, 2H, H2′, H6′), 6.65 (s, 1H, H3), 6.76(s, 1H, H6), 6.81 (s, 1H, H7), 7.98 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 16.3 (NCH_2CH_3), 46.1 (C7′), 46.5 (C8′), 52.2 (C9′-OCH_3), 55.4 (NCH_2CH_3), 56.0 (C3′-OCH_3, C5′-OCH_3), 60.8 (C4′-OCH_3), 101.3 (C10), 104.7 (C2′, C6′), 107.9 (C6), 109.7 (C3), 126.5 (C1), 131.5 (C8), 132.1 (C2), 135.2 (C7), 136.7 (C4′), 138.2 (C1′), 147.0 (C5), 148.3 (C4), 153.0 (C3′, C5′), 160.7 (C9), 173.0 (C9′). IR (film) cm^{-1}: 1733 ($COOCH_3$), 1623 (C═N). HRMS: calcd for $C_{25}H_{27}NO_7$, 453.1788; found, 453.1804. [α]$^{22}_D$ −159° (*c* 0.63%). UV (EtOH) λ_{max} 212 (lg ε 4.5), 246 (lg ε 4.4), 341 (lg ε 4.2). Anal. ($C_{25}H_{27}NO_7$) C, H, N.

9-Ethylimine of Methyl 9-Deoxy-6-methoxy-9-oxo-α-apopicropodophyllate (6). From aldehyde **4** (18 mg, 0.042 mmol), anhydrous $MgSO_4$ (20 mg, 0.16 mmol), and ethylamine 70% in water (16 μL, 0.20 mmol) using method A during 10 d. ^{1}H NMR ($CDCl_3$): δ 1.16 (t, 3H, J = 7.3 Hz, NCH_2CH_3), 3.50 (m, 2H, NCH_2CH_3), 4.06 (s, 3H, C6OCH_3), 6.39 (s, 1H, H3), 7.21 (s, 1H, H7), 8.01 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 16.3 (NCH_2CH_3), 55.3 (NCH_2CH_3), 59.8 (C2-OCH_3), 104.0 (C5), 118.6 (C1), 129.7 (C7), 131.1 (C8), 132.5 (C6), 135.0 (C3), 140.0 (C2), 149.8 (C4), 161.2 (C9). IR (film) cm^{-1}: 1732 ($COOCH_3$), 1633 (C═N). HRMS: calcd for $C_{26}H_{29}NO_8$+H, 484.1971; found, 484.1965.

9-Propylimine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (7). From the aldehyde **2** (82 mg, 0.19 mmol), anhydrous $MgSO_4$ (47 mg, 0.38 mmol), and propylamine (16 μL, 0.19 mmol) using method A during 7 d. ^{1}H NMR ($CDCl_3$): δ 0.80 (t, 3H, J = 7.3 Hz, $NCH_2CH_2CH_3$), 1.56 (m, 2H, $NCH_2CH_2CH_3$), 3.50 (m, 2H, $NCH_2CH_2CH_3$), 6.79 (s, 1H, H7), 7.93 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 11.5 ($NCH_2CH_2CH_3$), 23.9 ($NCH_2CH_2CH_3$), 62.9 ($NCH_2CH_2CH_3$), 135.0 (C7), 161.0 (C9). IR (film) cm^{-1}: 1732 ($COOCH_3$), 1623 (C═N). HRMS: calcd for $C_{26}H_{29}NO_7$+H, 468.2022; found, 468.2023. Anal. ($C_{26}H_{29}NO_7$) C, H, N.

9-*n*-Hexylimine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (8). From the aldehyde **2** (100 mg, 0.235 mmol), *n*-hexylamine (30 μL, 0.22 mmol), and molecular sieves 0.4 nm (100 mg) in CH_2Cl_2 (15 mL) using method B during 14 d. After purification, the imine **8** was obtained (111 mg, 93%). ^{1}H NMR ($CDCl_3$): δ 0.80−1.60 (m, 11H, $NCH_2C_5H_{11}$), 3.45 (m, 2H, $NCH_2C_5H_{11}$), 6.80 (s, 1H, H7), 7.95 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 14.1 ($NC_5H_{10}CH_3$), 22.6 ($NC_4H_8CH_2CH_3$), 26.8 ($NC_2H_4CH_2C_3H_7$), 30.9 ($NC_3H_6CH_2C_2H_5$), 31.6 ($NCH_2CH_2C_4H_9$), 61.3 ($NCH_2C_5H_{11}$), 135.2 (C7), 161.1 (C9). IR (film) cm^{-1}: 1732 ($COOCH_3$), 1622 (C═N). HRMS: calcd for $C_{29}H_{35}NO_7$, 509.2414; found, 509.2416. [α]$^{22}_D$ −156° (*c* 1.03%). UV (EtOH) λ_{max} 208 (lg ε 4.5), 243 (lg ε 4.3), 340 (lg ε 4.1).

9-Cyclohexylimine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (9). From the aldehyde **2** (100 mg, 0.235 mmol), cyclohexylamine (26 μL, 0.22 mmol), and molecular sieves 0.4 nm (100 mg) in C_6H_6 (15 mL) using method B during 7 d. After purification, the imine **9** was obtained (108 mg, 91%). ^{1}H NMR ($CDCl_3$): δ 1.20−1.90 (m, 10H, $NCH(C_5H_{10})$), 3.04 (m, 1H, $NCH(C_5H_{10})$), 6.74 (s, 1H, H7), 7.97 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 24.6 (2C, $NCH(C_5H_{10})$), 25.7 (1C, $NCH(C_5H_{10})$), 34.3 (2C, $NCH(C_5H_{10})$), 69.2 ($NCH(C_5H_{10})$), 134.8 (C7), 158.5 (C9). IR (film) cm^{-1}: 1732 ($COOCH_3$), 1618 (C═N). HRMS: calcd for $C_{29}H_{33}NO_7$, 507.2257; found, 507.2264. [α]$^{22}_D$ −176° (*c* 1.01%). UV (EtOH) λ_{max} 209 (lg ε 4.4), 247 (lg ε 4.2), 340 (lg ε 4.0).

9-(2-Hydroxyethyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (10). From the aldehyde **2** (44 mg, 0.10 mmol), anhydrous $MgSO_4$ (26 mg, 0.21 mmol), and ethanolamine (10 μL, 0.17 mmol) using method A during 4 d. ^{1}H NMR ($CDCl_3$): δ 3.42−3.80 (m, 4H, NC_2H_4OH), 6.87 (s, 1H, H7), 8.05 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 62.2 (NCH_2CH_2OH), 62.6 (NCH_2CH_2OH), 136.3 (C7), 163.4 (C9). IR (film) cm^{-1}: 3600−3100 (OH), 1726 ($COOCH_3$). HRMS: calcd for $C_{25}H_{27}NO_8$, 469.1737; found, 469.1735. [α]$^{22}_D$ −188° (*c* 0.71%). UV (EtOH) λ_{max} 206 (lg ε 4.4), 247 (lg ε 4.1), 314 (lg ε 3.7), 342 (lg ε 3.9).

9-Ethylendiimine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (11). The aldehyde **2** (64 mg, 0.15 mmol), anhydrous $MgSO_4$ (36 mg, 0.30 mmol), and ethylenediamine (30 μL, 0.45 mmol) were stirred at room temperature during 20 h. The mixture was processed following method A to yield the diimine **11** (65 mg, 99%). ^{1}H NMR ($CDCl_3$): δ 3.42−3.80 (m, 4H, NC_2H_4N), 6.73 (s, 4H, H5, H7), 7.74 (s, 2H, H9); ^{13}C NMR ($CDCl_3$): δ 60.4 (NCH_2CH_2N), 60.8 (NCH_2CH_2N), 135.8 (C7), 163.2 (C9). IR (film) cm^{-1}: 1732 ($COOCH_3$), 1622 (C═N). HRMS: calcd for $C_{48}H_{48}N_2O_{14}$+H, 877.3184; found, 877.3272.

9-(2-Aminoethyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (12). To a mixture of ethylenediamine (0.50 mL, 7.5 mmol) and anhydrous $MgSO_4$ (37 mg, 0.30 mmol) in CH_2Cl_2 (1.0 mL) was added a solution of the aldehyde **2** (44 mg, 0.10 mmol) in CH_2Cl_2 (1.0 mL). The reaction was stirred at room temperature during 40 min and filtered and the filtrate concentrated under vacuum. The product **12** (48 mg, 99%) was obtained. ^{1}H NMR ($CDCl_3$): δ 2.86 (m, 4H, $NC_2H_4NH_2$), 6.81 (s, 1H, H7), 7.98 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 42.4 ($NCH_2CH_2NH_2$), 63.9 ($NCH_2CH_2NH_2$), 135.7 (C7), 162.4 (C9). IR (film) cm^{-1}: 3400−3200 (NH_2), 1731 ($COOCH_3$), 1621 (C═N). HRMS: calcd for $C_{25}H_{28}N_2O_7$+H, 469.1974; found, 469.1943.

9-Phenylimine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (13). From the aldehyde **2** (67 mg, 0.16 mmol), anhydrous $MgSO_4$ (39 mg, 0.32 mmol) and freshly distilled aniline (15 μL, 0.16 mmol) using method A during 30 d. ^{1}H NMR ($CDCl_3$): δ 7.03 (s, 1H, H7), 7.20 (m, 5H, NC_6H_5), 8.21 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 121.0 (2C, NC_6H_5), 125.9 (1C, NC_6H_5), 129.1 (2C, NC_6H_5), 138.1 (C7), 153.1 (1C, NC_6H_5), 160.2 (C9). IR (film) cm^{-1}: 1734 ($COOCH_3$). HRMS: calcd for

$C_{29}H_{27}NO_7$, 501.1788; found, 501.1791. Anal. ($C_{29}H_{27}NO_7$) C, H, N. UV (EtOH) λ_{max} 209 (lg ϵ 5.4), 256 (lg ϵ 4.4), 362 (lg ϵ 4.3).

9-(4-Methoxyphenyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (14). From the aldehyde **2** (121 mg, 0.28 mmol), anhydrous $MgSO_4$ (24 mg, 0.20 mmol) and *p*-anisidine (34 mg, 0.28 mmol) in CH_2Cl_2 (2.0 mL) using method A during 11 d. ^{1}H NMR ($CDCl_3$): δ 3.78 (s, 3H, $NC_6H_4OCH_3$), 6.87 (d, 2H, J = 8.8 Hz, $NC_6H_4OCH_3$), 6.99 (s, 1H, H7), 7.13 (d, 2H, J = 8.8 Hz, $NC_6H_4OCH_3$), 8.23 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 55.2 ($NC_6H_4OCH_3$), 114.1 (2C, $NC_6H_4OCH_3$), 122.1 (2C, $NC_6H_4OCH_3$), 136.9 (C7), 144.3 (1C, $NC_6H_4OCH_3$), 158.0 (1C, $NC_6H_4OCH_3$), 157.9 (C9). IR (film) cm^{-1}: 1731 ($COOCH_3$), 1607 (C=N). HRMS: calcd for $C_{30}H_{29}NO_8$+H, 532.1971; found, 532.1986.

9-(3,4,5-Trimethoxyphenyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (15). From the aldehyde **2** (59 mg, 0.14 mmol), anhydrous $MgSO_4$ (34 mg, 0.28 mmol), and 3,4,5-trimethoxyaniline (39 mg, 0.21 mmol) in CH_2Cl_2 (2.0 mL) using method A during 14 d. After evaporating the solvent, the reaction product was chromatographed on silica gel, eluting with CH_2Cl_2, to give the compound **15** (78 mg, 95%). ^{1}H NMR ($CDCl_3$): δ 3.83 (s, 3H, $NC_6H_2(OCH_3)_3$), 3.86 (s, 6H, $NC_6H_2(OCH_3)_3$), 6.37 (s, 2H, $NC_6H_2(OCH_3)_3$), 7.06 (s, 1H, H7), 8.22 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 56.1 (2C, $NC_6H_2(OCH_3)_3$), 61.0 (1C, $NC_6H_2(OCH_3)_3$), 98.3 (2C, $NC_6H_2(OCH_3)_3$), 136.4 (1C, $NC_6H_2(OCH_3)_3$), 138.1 (C7), 147.2 (1C, $NC_6H_2(OCH_3)_3$), 153.5 (2C, $NC_6H_2(OCH_3)_3$), 159.5 (C9). IR (film) cm^{-1}: 1731 ($COOCH_3$). HRMS: calcd for $C_{32}H_{33}NO_{10}$, 591.2104; found, 591.2106. $[\alpha]^{22}_D$ − 309° (*c* 0.125%). UV (EtOH) λ_{max} 206 (lg ϵ 4.7), 256 (lg ϵ 4.3), 371 (lg ϵ 4.3).

9-(2-Hydroxyphenyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (16). From the aldehyde **2** (42 mg, 0.10 mmol), aanhydrous $MgSO_4$ (34 mg, 0.28 mmol), and 2-aminophenol (11 mg, 0.10 mmol) in dry EtOH (3.0 mL) using method C during 24 h. ^{1}H NMR ($CDCl_3$): δ 6.90 (m, 2H, NC_6H_4OH), 7.08 (s, 1H, H7), 7.16 (m, 2H, NC_6H_4OH), 8.42 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 114.9 (1C, NC_6H_4OH), 115.3 (1C, NC_6H_4OH), 119.9 (1C, NC_6H_4OH), 128.7 (1C, NC_6H_4OH), 134.9 (1C, NC_6H_4OH), 138.7 (C7), 152.3 (1C, NC_6H_4OH), 156.3 (C9). IR (film) cm^{-1}: 3500−3200 (OH), 1731 ($COOCH_3$). HRMS: calcd for $C_{29}H_{27}NO_8$+H, 518.1815; found, 518.1869.

9-(2-Hydroxy-5-methylphenyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (17). From the aldehyde **2** (43 mg, 0.10 mmol), anhydrous $MgSO_4$ (49 mg, 0.40 mmol), and 2-amino-*p*-cresol (13 mg, 0.10 mmol) using method A during 24 h. The crude yield of imine was over 90%, but this was contaminated with the starting aldehyde. Removal of the starting aldehyde by chromatography on a silica gel column eluting with 2% EtOAc/CH_2Cl_2 gave the pure imine **17** (13 mg, 24%). ^{1}H NMR ($CDCl_3$): δ 2.27 (s, 3H, $NC_6H_3(OH)CH_3$), 6.83 (d, 1H, J = 8.0 Hz, $NC_6H_3(OH)CH_3$), 6.97 (dd, 1H, J = 1.8 Hz, $NC_6H_3(OH)CH_3$), 7.01 (d, 1H, J = 1.8 Hz, $NC_6H_3(OH)CH_3$), 7.07 (s, 1H, H7), 8.41 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 20.8 ($NC_6H_3(OH)CH_3$), 114.6 (1C, $NC_6H_3(OH)CH_3$), 115.8 (1C, $NC_6H_3(OH)CH_3$), 129.2 (1C, $NC_6H_3(OH)CH_3$), 129.4 (1C, $NC_6H_3(OH)CH_3$), 134.6 (1C, $NC_6H_3(OH)CH_3$), 138.5 (C7), 150.2 (1C, $NC_6H_3(OH)CH_3$), 156.0 (C9). IR (film) cm^{-1}: 3500−3200 (OH), 1728 ($COOCH_3$). HRMS: calcd for $C_{30}H_{29}NO_8$+H, 532.1971; found, 532.2012.

9-(5-Chloro-2-hydroxyphenyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (18). From the aldehyde **2** (49 mg, 0.11 mmol), anhydrous $MgSO_4$ (56 mg, 0.46 mmol), and 2-amino-4-chlorophenol (17 mg, 0.11 mmol) using method A during 3 d. The crude yield of imine was over 90%, but this was contaminated with the starting aldehyde. Removal of the starting aldehyde by chromatography on a silica gel column eluting with 2% EtOAc/CH_2Cl_2 gave the pure imine **18** (41 mg, 65%). ^{1}H NMR ($CDCl_3$): δ 6.87 (d, 1H, J = 8.8 Hz, NC_6H_3ClOH), 7.09 (dd, 1H, J = 8.8, 2.4 Hz, NC_6H_3ClOH), 7.11 (s, 1H, H7), 7.17 (d, 1H, J = 2.4 Hz, NC_6H_3ClOH), 8.36 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 115.7 (1C, NC_6H_3ClOH), 115.9 (1C, NC_6H_3ClOH), 124.8 (1C, NC_6H_3ClOH), 128.2 (1C, NC_6H_3ClOH), 135.8 (1C, NC_6H_3ClOH), 139.8 (C7), 150.9 (1C, NC_6H_3ClOH), 157.5 (C9). IR (film) cm^{-1}: 3500−3200 (OH), 1727 ($COOCH_3$). HRMS: calcd for $C_{29}H_{26}NO_8Cl$+H, 522.1425; found, 552.1438. Anal. ($C_{29}H_{26}NO_8Cl$) C, H, N.

9-(2-Amino-4,5-dimethylphenyl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (19). From the aldehyde **2** (69 mg, 0.16 mmol), anhydrous $MgSO_4$ (59 mg, 0.49 mmol), and 4,5-dimethyl-*o*-phenylendiamine (22 mg, 0.16 mmol) using method A during 5 d. The crude yield of imine was over 90%, but this was contaminated with the starting aldehyde. Removal of the starting aldehyde by chromatography on a silica gel column eluting with 10% EtOAc/CH_2Cl_2 gave the pure imine **19** (41 mg, 47%). ^{1}H NMR ($CDCl_3$): δ 2.15 (s, 3H, $NC_6H_2(CH_3)_2NH_2$), 2.16 (s, 3H, $NC_6H_2(CH_3)_2NH_2$), 6.53 (s, 1H, $NC_6H_2(CH_3)_2NH_2$), 6.80 (s, 1H, $NC_6H_2(CH_3)_2NH_2$), 6.98 (s, 1H, H7), 8.29 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 18.9 ($NC_6H_2(CH_3)_2NH_2$), 19.5 ($NC_6H_2(CH_3)_2NH_2$), 116.9 (1C, $NC_6H_2(CH_3)_2NH_2$), 117.4 (1C, $NC_6H_2(CH_3)_2NH_2$), 126.1 (1C, $NC_6H_2(CH_3)_2NH_2$), 134.0 (1C, $NC_6H_2(CH_3)_2NH_2$), 136.3 (1C, $NC_6H_2(CH_3)_2NH_2$), 136.4 (C7), 140.4 (1C, $NC_6H_2(CH_3)_2NH_2$), 155.1 (C9). IR (film) cm^{-1}: 3500−3200(NH_2), 1730($COOCH_3$). HRMS: calcd for $C_{31}H_{32}N_2O_7$+H, 545.2287; found, 545.2297.

9-(4-Aminopyrimidin-5-yl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (20). From the aldehyde **2** (108 mg, 0.25 mmol), anhydrous $MgSO_4$ (124 mg, 1.02 mmol), and 4,5-diaminopyrimidine (30 mg, 0.26 mmol) in dry EtOH (3.0 mL), using method C during 4 d. ^{1}H NMR ($CDCl_3$): δ 7.11 (s, 1H, H7), 7.97 (s, 1H, $NC_4H_2N_2NH_2$), 8.28 (s, 1H, H9), 8.39 (s, 1H, $NC_4H_2N_2NH_2$); ^{13}C NMR ($CDCl_3$): δ 129.7 (1C, $NC_4H_2N_2NH_2$), 139.8 (C7), 140.2 (1C, $NC_4H_2N_2NH_2$), 156.0 (1C, $NC_4H_2N_2NH_2$), 158.6 (1C, $NC_4H_2N_2NH_2$), 160.0 (C9). IR (film) cm^{-1}: 3500−3200 (NH_2), 1728 ($COOCH_3$), 1613 (C=N). HRMS: calcd for $C_{27}H_{26}N_4O_7$+H, 519.1879; found, 519.1932.

9-(1-Ethylpyrazol-5-yl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (21). From the aldehyde **2** (100 mg, 0.235 mmol) and 5-amino-1-ethylpyrazole (39 mg, 0.35 mmol) in dry C_6H_6 (10 mL) using the method D during 2 d. The crude yield of imine was over 90%, but this was contaminated with the starting aldehyde. Removal of the starting aldehyde by chromatography on a silica gel column eluting with 20% EtOAc/CH_2Cl_2, gave the pure imine **21** (40 mg, 33%). ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 3H, J = 7.3 Hz, NCH_2CH_3), 4.18 (q, 1H, J = 7.3 Hz, NCH_2CH_3), 4.25 (q, 1H, J = 7.3 Hz, NCH_2CH_3), 6.11 (d, 1H, J = 2.0 Hz, $NC_3H_2N_2$-Et), 7.08 (s, 1H, H7), 7.42 (d, 1H, J = 2.0 Hz, $NC_3H_2N_2$-Et), 8.32 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 15.4 (NCH_2CH_3), 42.8 (NCH_2CH_3), 91.4 (1C, $NC_3H_2N_2$-Et), 138.5 (1C, $NC_3H_2N_2$-Et), 138.8 (C7), 147.2 (1C, $NC_3H_2N_2$-Et), 159.2 (C9). IR (film) cm^{-1}: 1731 ($COOCH_3$). HRMS: calcd for $C_{28}H_{29}N_3O_7$+H, 520.2084; found, 520.2028. Anal. ($C_{28}H_{29}N_3O_7$) C, H, N.

9-(Thiazol-2-yl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (22). From the aldehyde **2** (100 mg, 0.235 mmol) and 2-aminothiazole (242 mg, 2.35 mmol) in dry CH_6Cl_6 (25 mL) using method D during 3 d. The crude yield of imine was over 90%, but this was contaminated with the starting aldehyde. Removal of the starting aldehyde by chromatography on a silica gel column basified with Et_3N eluting with 1% Et_3N/CH_2Cl_2, gave the pure imine **22** (64 mg, 57%). ^{1}H NMR ($CDCl_3$): δ 7.16 (d, 1H, J = 3.7 Hz, NC_3H_2SN), 7.22 (s, 1H, H7), 7.61 (d, 1H, J = 3.7 Hz, N C_3H_2SN), 8.72 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 108.9 (1C, NC_3H_2SN), 117.8 (1C, NC_3H_2SN), 136.3 (C7), 163.3 (C9), 191.3 (1C, NC_3H_2SN). IR (film) cm^{-1}: 1732 ($COOCH_3$). HRMS: calcd for $C_{26}H_{24}N_2O_7S$, 508.1304; found, 508.1302. $[\alpha]^{22}_D$ − 95° (*c* 0.33%). UV (EtOH) λ_{max} 209 (lg ϵ 4.6), 257 (lg ϵ 4.2), 319 (lg ϵ 3.8), 391 (lg ϵ 4.2).

9-(5-Methylisoxazol-3-yl)imine of Methyl 9-Deoxy-9-oxo-α-apopicropodophyllate (23). From the aldehyde **2** (47 mg, 0.11 mmol) and 3-amino-5-methylisoxazole (43 mg, 0.44 mmol) in dry C_6H_6 (12 mL) using method D during 4 d. The crude yield of imine was over 90%, but this was contaminated with the starting aldehyde. Removal of the starting aldehyde by chromatography on a silica gel column eluting with 10% EtOAc/CH_2Cl_2 gave the pure imine **23** (50 mg, 90%). ^{1}H NMR

($CDCl_3$): δ 2.39 (s, 3H, $NC_3HONC\mathit{H}_3$), 6.00 (s, 1H, $NC_3\mathit{H}ONCH_3$), 7.13 (s, 1H, H7), 8.45 (s, 1H, H9); ^{13}C NMR ($CDCl_3$): δ 12.7 ($NC_3HONC\mathit{H}_3$), 96.0 (1C, NC_3HONCH_3), 141.0 (C7), 169.7 (1C, $N\mathit{C}_3HONCH_3$), 165.1 (C9), 170.4 (1C, NC_3HONCH_3). IR (film) cm^{-1}: 1731 ($COOCH_3$).

Biological Activity. Reagents. MEM, DMEM and RPMI 1640, fetal calf serum (FCS), antibiotics, and L-glutamine were purchased from Life Technologies, Inc. (Gaithersburg, MD). Rabbit polyclonal antiserum against human caspase-3 was from PharMingen (San Diego, CA). Mouse monoclonal antibody C2.10 against human poly(ADP-ribose) polymerase (PARP) was purchased from Enzyme Systems Products (Livermore, CA). Acrylamide, bisacrylamide, ammonium persulfate, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were from Bio-Rad (Richmond, CA). All other chemicals were from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cytotoxic Assays. A screening method previously described[27] was used to assess the antitumor activity against the following cell lines: P-388 (lymphoid neoplasma from DBA/2 mouse), A-549 (human lung carcinoma), HT-29 (human colon carcinoma), and MEL-28 (human melanoma). 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 MEM−10% FCS medium containing the compound to be evaluated at different concentrations. In each case, a set of control wells was incubated in the absence of drug and counted daily to ensure exponential cell growth. After 4 days at 37 °C, under a 10% CO_2, 98% humid atmosphere, P-388 cells were observed by inverted microscopy and the degree of inhibition was determined by comparison with the controls, while A-549, HT-29, and MEL-28 were stained with crystal violet before examination. All calculations represent the average of duplicate wells.

Analysis of Cell Cycle and Apoptosis by Flow Cytometry. For cell cycle analysis, we used the human epitheloid cervix adenocarcinoma HeLa cell line and the human colon adenocarcinoma HT-29 cell line grown in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 100 units/mL penicillin, and 24 μg/mL gentamicin and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Untreated and drug-treated cells ($(3-5) \times 10^5$) were centrifuged and fixed overnight in 70% ethanol at 4 °C. Then, cells were washed three times with PBS, incubated for 1 h with 1 mg/mL RNAse A and 20 μg/mL propidium iodide at room temperature, and analyzed with a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA) as described previously.[30,31] The induction of apoptosis was monitored as the appearance of the sub-G_1 peak in cell cycle analysis.[30,31] Quantification of apoptotic cells was calculated as the percentage of cells in the sub-G_1 region (hypodiploidy) in cell cycle analysis.

Western Blot Analysis. About 10^7 cells were pelleted by centrifugation, washed with PBS, lysed, and subjected to Western blot analysis as described previously.[32] Proteins (20 μg) were separated through sodium dodecyl sulfate−polyacrylamide gels under reducing conditions, transferred to nitrocellulose filters, blocked with 5% powder defatted milk, and incubated overnight with the corresponding antibodies. Signals were developed using an enhanced chemiluminescence (ECL) detection kit (Amersham, Aylesbury, UK).

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Supporting Information Available: Complete ^1H and ^{13}C NMR data for all synthesized analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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