Antitumor Agents. 207.[1] Design, Synthesis, and Biological Testing of 4β-Anilino-2-fluoro-4′-demethylpodophyllotoxin Analogues as Cytotoxic and Antiviral Agents

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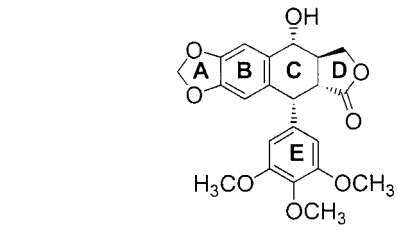
2-Fluoropodophyllotoxin (**11**) and several 4β-anilino-2-fluoro-4′-*O*-demethyl analogues were synthesized and evaluated in both antineoplastic and antiviral assays. These compounds were moderately active against some cancer cell lines, but they were less active than the corresponding nonfluorinated analogues. Compound **11** exhibited the best activity against KB carcinoma with a GI_{50} of approximately 30 nM. Most compounds exhibited moderate activity against HCMV with ID_{50} and ID_{90} values in the range of 1 μM and 4 μM, respectively. Both **9** and **11** showed an unusual 10-fold selectivity for HSV-2 compared to HSV-1.

Introduction

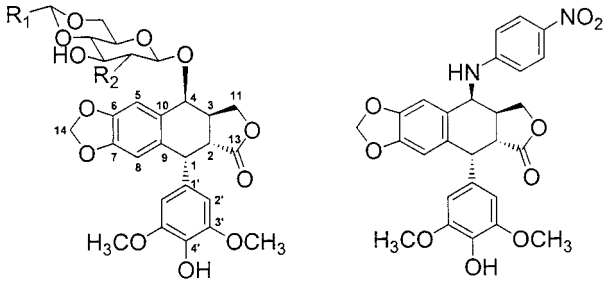
Podophyllum pelatum L. (and *P. emodi L.*, found in China and India), also known as the American Mandrake or Mayapple, is the source of the natural product podophyllotoxin (**1**). Its medicinal properties have been well-recognized for centuries. The first written account of its medical value occurred in 1731 in *The Natural History of the Carolina's*, where this plant is described as an emetic and a purgative. From 1820 to 1942, *P. pelatum L.* was listed in the United States Pharmacopeia as a cathartic and a cholagogue, but in 1942, it was removed from the 11th edition due to its undesirable toxicity. However, it is still listed in the Pharmacopeias of several foreign countries, including England, France, Germany, Norway, and Russia. Podophyllotoxin itself was first isolated in either 1880 or 1891, while its structure was elucidated in 1932. In 1952, the originally assigned stereochemistry was corrected to give the currently accepted structure shown in Chart 1.

The biological activity of **1** has led to extensive structural modification resulting in several clinically useful compounds. Etoposide (**2**)[2] and teniposide (**3**),[3] developed in the late 1960s and early 1970s by Sandoz, are two epipodophyllotoxins[4] in clinical use as antineoplastic agents. They are used against a variety of cancers, including germ-cell malignancies, small-cell lung cancer, non-Hodgkin's lymphoma, leukemia, Kaposi's sarcoma, neuroblastoma, and soft tissue sarcoma. In another podophyllum glycoside derivative, NK611 (**4**), the 2″-hydroxyl group in the glucose ring of **2** has been replaced with an *N*,*N*-dimethylamino moiety. The main advantage of this compound is its superior water solubility compared to that of **2**. It is currently undergoing phase I clinical evaluation, and further development in phase II is expected.[5] GL331 (**5**), which was developed in our laboratory in the early 1990s and licensed to Genelabs Technologies, Inc., contains a *p*-nitroanilino moiety at the 4β-position instead of a glycoside. It is currently in phase II clinical trials, focusing on gastric carcinoma, colon cancer, and non-small-cell lung cancer. It has also shown positive results against malignancies resistant to **2**.[6] Etopophos, an etoposide prodrug in which a disodium phosphate salt was prepared at the 4′-phenolic oxygen, is also in phase II clinical trials.[7]

Chart 1. Structures of Podophyllotoxin (**1**), Etoposide (**2**), Teniposide (**3**), NK-611 (**4**), and GL331 (**5**)



Podophyllotoxin (**1**)



Etoposide (**2**) $R_1 = CH_3$, $R_2 = OH$
Teniposide (**3**) R_1 = 2-thienyl, $R_2 = OH$
NK-611 (**4**) $R_1 = CH_3$, $R_2 = NMe_2$

GL331 (**5**)

Several different mechanisms of action have been connected to this compound family. Podophyllotoxin (**1**) inhibits tubulin polymerization through interaction at the colchicine binding site on the tubulin monomer. While the binding of **1** to tubulin is slow (1000/M·s at 37 °C) and has a high activation energy (14.7 kcal/mol), the affinity is relatively low (1.8 μM at 37 °C).[8] Demethylation of the 4′-methoxy moiety eliminates the

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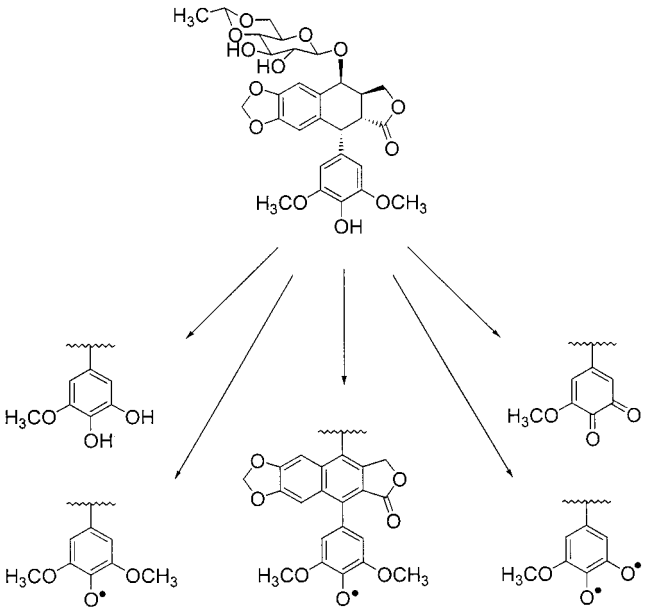
Chart 2. Oxidative Metabolic Pathway for Podophyllotoxin



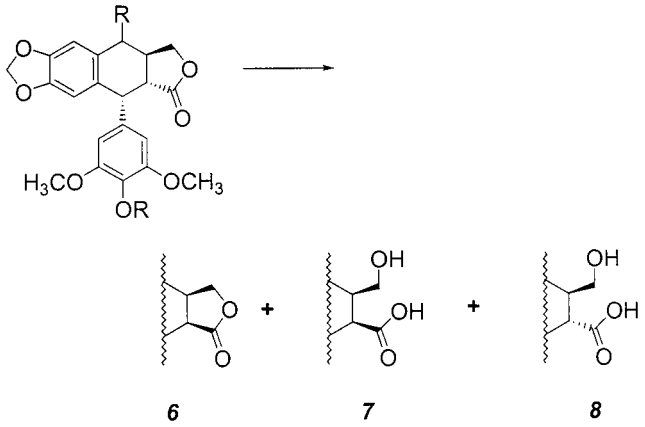
antimitotic activity of these compounds. Etoposide and other related compounds are potent inhibitors of DNA topoisomerase II (topo II). Topo II is an essential enzyme that functions in the segregation of newly replicated chromosome pairs, in chromosome condensation, and in alteration of DNA supercoiling.[9] GL331, which is also an inhibitor of topo II, has recently been connected to apoptotic cell death via activation of CDC2 kinase.[10] Another enzyme, CDC25, is a dual phosphatase responsible for the dephosphorylation of cyclin B-associated CDC2 in the G_2 stage of mitosis. This dephosphorylation activates CDC2, which has been previously implicated in apoptotic cell death.[11] One study indicates that GL331 facilitates the association between CDC25 and Raf-1. This interaction stimulates the downstream signaling pathways of Raf-1, which includes the CDC2 dephosphorylation, causing apoptosis in treated cells.

Due to the rich variety of biological activity found in the podophyllum family of compounds, numerous studies have been performed to determine the routes of metabolism in vivo. Two main pathways have been found: an oxidative pathway, which is also important for the mechanism of action, and a hydrolytic pathway, which leads directly to inactive compounds.

The oxidative pathway for **2** and **3** is shown in Chart 2.[12] Part of or this entire route will likely apply to other related compounds. Etoposide-induced DNA strand cleavage requires the presence of metal ions (specifically Cu^{2+} and Fe^{3+}).[13] This cleavage can also be induced by irradiation with UV light and inhibited with known radical scavengers. Using ESR spin-trapping techniques, Sakurai et al. were able to conclusively detect the formation of a hydroxy (not phenoxy) radical. This result suggests that the active species for DNA cleavage is a hydroxy radical formed by metal/drug-induced activation of a water molecule. SAR studies have indicated that the 4′-phenol also is important for this activity.[14]

Three major hydrolytic metabolites have been found for **1**−**3**: the picro form (**6**), the *cis*-hydroxy acid (**7**), and the *trans*-hydroxy acid (**8**) (see Chart 3). All of these

Chart 3. Hydrolytic Metabolism for Podophyllium Compounds

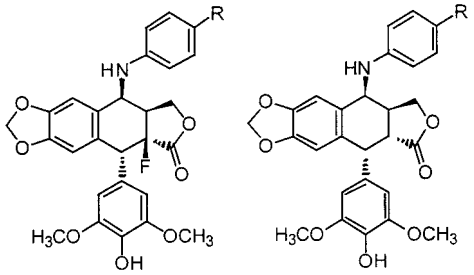


metabolites are inactive as antitumor agents.[15] In rat livers isolated after tritium-labeled etoposide administration, 90% of radiolabel was found in the bile within 3 h of administration.[16] Studies in cancer patients following intravenous administration of radiolabeled **2** detected up to 19% of the label in the bile and 58% in the urine.[17] In both studies, the compounds were confirmed either as **6**, **7**, or **8** by HPLC analysis. The C-2 hydrogen can be removed chemically at pH 9.3 to give the enolate. Subsequent reprotonation gives the thermodynamically more stable picro form.[18] At pH 12, the lactone ring opens to give the hydroxy acids, and the equilibrium shifts back to the lactone when the pH drops below 4.[11]

Several different approaches have been tried to ameliorate the problem of hydrolytic inactivation. One method involves replacing the C-2 hydrogen atom in a diastereospecific manner with a functionality that is not susceptible to enolization. Glinski and co-workers prepared a series of C-2 substituted derivatives where the hydrogen had been replaced with a series of small functional groups.[19] The enolate was formed using LDA in dry THF and quenched in a diastereospecific manner with various electrophilic groups. Using this procedure, the 2-methyl, 2-chloro, 2-bromo, 2-(methylthio), and 2-hydroxyl compounds were prepared. Only the chloro derivative showed significant activity in the P388 and L1210 assays, giving a T/C of 156 against P388 leukemia at 40 mg/kg dosing.

A second method attempted to inhibit metabolic inactivation was replacement of the C-2 carbon with a nitrogen atom.[20] Several derivatives of the 2-aza compounds were prepared and tested for biological activity. They were all active against KB carcinoma; however, they were less active than **2**.[21] Many other D-ring modified derivatives of the epipodophyllotoxins have been prepared, including D-ring cyclopentanone, cyclopentane, ether, sulfide, sulfone, and sulfoxide analogues. All of these derivatives were found to be less active than their parent compounds in mitotic inhibition and cytotoxicity assays.[22,23] Clinical evaluation was begun but discontinued on two other derivatives due to a poor activity profile and unacceptable cytotoxicity.[24] The activity found with 2-chloroetoposide initiated an attempt to prepare the corresponding 2-fluoro derivative. Unfortunately, at that time, the current electrophilic fluorinating reagents were precarious. One reported

Table 1. Electrostatic and Mulliken Charge Comparisons for the Lactone Carbonyl Carbon (C-13)



	2-F derivatives		2-H derivatives	
R group	electrostatic charge	Mulliken charge	electrostatic charge	Mulliken charge
F	0.88	0.35	0.86	0.38
CN	0.87	0.35	0.89	0.38
NO_2	0.87	0.35	0.86	0.38
3,4-OCH_2CH_2O	0.88	0.35	0.90	0.38
NH_2	0.89	0.35	0.87	0.38
DMEP[a]	1.09	1.00	1.12	0.99

[a] This compound was optimized with the 3-21G* basis set. All others were optimized using PM3.

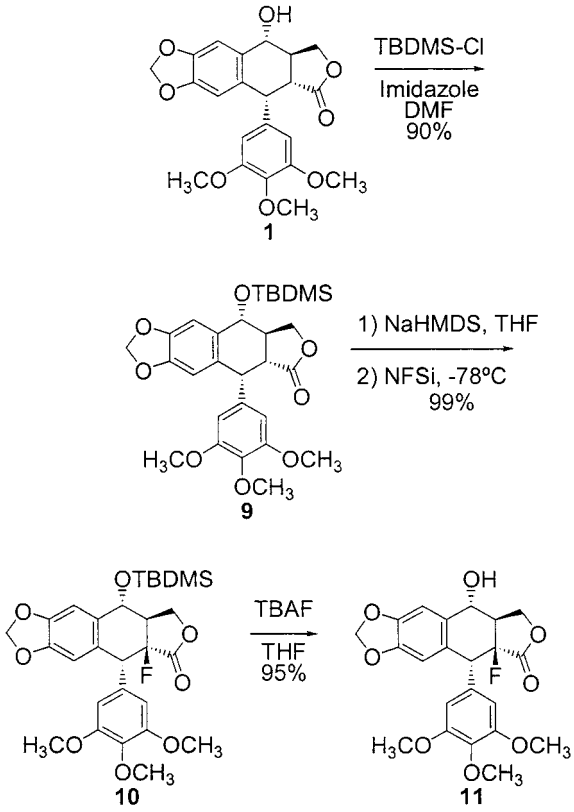
attempt using perchloryl fluoride ($FClO_4$, also known as fluoroperchlorate) in LDA resulted in a "violent explosion, causing serious injury".[19]

We have reported already on the diastereospecific synthesis of 2-fluoropodophyllotoxin.[25] In this report, we present the continued synthesis and examination of related fluorinated compounds. We have prepared several 4β-anilino-2-fluoro-4′-*O*-demethylpodophyllotoxins related to **5** and examined their biological activity in both antiviral and antineoplastic assays.

Calculations

Before attempting the synthesis of the 2-fluoropodophyllotoxin, we considered the possibility that, due to its electronegativity, fluorination might increase the partial positive charge at the carbonyl carbon and greatly increase the likelihood of hydrolysis of the D-ring lactone. This question was addressed computationally using both semiempirical and ab initio calculations. The geometries of the test compounds were optimized using both SYBYL and MacSpartan Plus software packages. A conformational search of all rotatable bonds was done using the systematic search option within SYBYL. The rotatable bonds were examined in 5° increments with a minimum energy cutoff of 999 kcal/mol, and all conformations were saved into a separate database. The structures then were optimized using the SYBYL force field with Gasteiger−Huckel charges. The five lowest-energy conformations were exported into MacSpartan Plus for optimization at either the PM3 or 3-21G* level. Compound **12** and its corresponding nonfluorinated analogue were optimized using 3-21G* while, due to a limit on the number of atoms permitted for the ab initio basis sets in MacSpartan Plus, the other compounds were optimized at the PM3 semiempirical level. The lowest-energy conformation was kept for each compound and all others were discarded. The electrostatic and Mulliken charges of the lactone carbonyl carbons were compared for the fluorinated and nonfluorinated derivatives. These results are shown in Table 1. Because of its high electronegativity, fluorine has a strong electron-withdrawing inductive effect; however, its several lone pairs of electrons can be donated via a mesomeric effect. In these compounds, these two effects nearly balance out, leading to a negligible difference for the partial positive charge on the carbonyl carbon between the fluorinated and nonfluorinated compounds at both theory levels. Although unexpected and difficult to explain, this result did not immediately counter-indicate the preparation of the fluorinated series of compounds.

Scheme 1. Preparation of 2-Fluoropodophyllotoxin (**11**)



Chemistry

The synthesis of 2-fluoropodophyllotoxin (**11**) is shown in Scheme 1.[25] Briefly, **1** was protected as the TBDMS ether (**9**) using standard conditions.[26] The electrophilic fluorination was performed using a combination of the methods of Davis[27] and Differding to give **10**.[28] The deprotection was carried out with tetrabutylammonium fluoride in THF to give the final product (**11**). The overall yield for the three steps was 85%.

Demethylation of the 4′-methoxy group was extremely difficult. Attempts to prepare 2-fluoro-4′-*O*-demethylepipodophyllotoxin (**12**) using standard conditions [HBr(g) in 1,2-dichloroethane followed by water, acetone, and $BaCO_3$] gave a complex mixture. Varying the reaction temperature from −40 to +20 °C did not change the composition. TLC analysis showed five or six spots in all cases. BBr_3 at varying temperatures (−78 to 0 °C) also gave a complex mixture. Although several spots were positive for a phenol,[29] a cleaner method was desired. Literature examination revealed that trimethylsilyl iodide (TMS-I) had been used successfully for the selective demethylation of podophyllotoxin.[30] Accordingly, 2-fluoropodophyllotoxin was selectively and cleanly demethylated using the procedure of Daley et al.

Scheme 2. Preparation of 4β-Anilino-2-fluoro-4′-demethylpodophyllotoxin Analogues

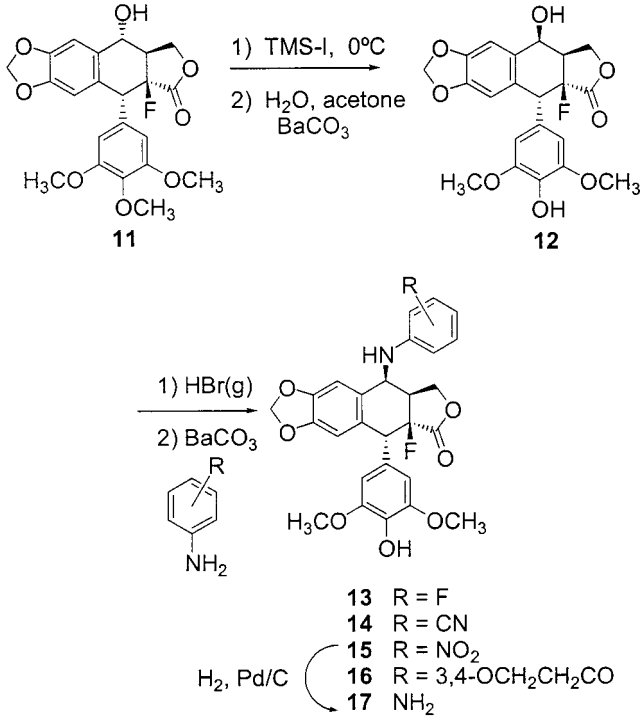


Table 2. Herpesvirus Activity for 2-Fluoropodophyllotoxin and Its Intermediates[a]

	cell growth inhib		HSV-1 inhib		HSV-2 inhib	
compd	ED_{25} (nM)	MIC (nM)	ED_{100} (nM)	MIC (nM)	ED_{100} (nM)	MIC (nM)
1	20	8	50	10	100	10
9	6000	3000	10000	1000	1000	100
10	25000	6000	NA	NA	NA	NA
11	1000	800	10000	1000	1000	100

[a] NA = not active; MIC = minimum inhibitory concentration.

(Scheme 2). The only observed product was **12.** The conversion was only 25% (based on recovered starting material); however, the yield was 80%. This procedure was selected because the starting material was readily recovered and recycled. Optimal purity and yields were obtained at 0 °C using 3.1 equiv of TMS-I. Selected side chains were added at position-4 based upon previous studies in our laboratory.[31] HBr(g) in 9:1 1,2-dichloroethane:diethyl ether was added to **12** at 0 °C to generate the secondary bromide. A substituted aniline and $BaCO_3$ were then added, and the reaction mixture stirred for 12 h. Flash chromatography gave the desired 4β-anilino compounds **13**−**16** in moderate to good yields. Compound **17** was prepared by catalytic hydrogenation of **15**. NMR analysis of the final products confirmed both the β-conformation of the C-4 anilino moiety and the *trans* C/D-ring fusion.

Results and Discussion

The in vitro antiviral and antineoplastic data are presented in Tables 2−5. The herpesvirus simplex 1 and 2 (HSV-1 and HSV-2) screening was done according to the procedure of Bastow et al.[32] The cell growth inhibition (column 1) measures the death of the healthy cells; the other columns indicate the inhibition of HSV-1 and HSV-2. 2-Fluoropodophyllotoxin (**11**) and its precursors (**9**, **10**) were less cytotoxic than the podophyllotoxin control. Compounds **9** and **11** were less active against both HSV-1 and HSV-2 and **10** was inactive. On comparing **1** and **11**, fluorination resulted in a 50-fold drop in cytotoxicity, but also a 200-fold drop in the ED_{100} against HSV-1 and a 10-fold drop against HSV-2. The minimum inhibitory concentration (MIC) also dropped similarly. Both **9** and **11** showed an unusual 10-fold selectivity for HSV-2 compared to HSV-1. The 4β-anilino-2-fluoro-4′-*O*-demethylpodophyllotoxins **12**−**17** were not examined in this assay, because 2-fluoropodophyllotoxin displayed greatly decreased activity and 4β-anilino compounds normally lack HSV activity.[33] In addition, etoposide is a nonselective inhibitor of HSV,[34]

Table 3. HCMV Screening Data for 4β-Anilino-2-fluoropodophyllotoxins

	2-F compd		2-H compd	HEL cells	
R group[a]	ID_{50} (μM)	ID_{90} (μM)	ID_{50} (μM)	ED_{50} (μM)	SI[b]
13: 4-F	0.97	4.47	0.65	6.10	6.2
14: 4-CN	0.76	4.29	0.62	8.43	11.1
15: 4-NO_2	1.45	4.60	0.35	6.09	4.2
16: 3,4-OCH_2CH_2CO	1.30	2.31		6.70	5.2
17: 4-NH_2	2.54	5.02	1.25	7.50	3.0
11	>5	>5	<0.10[c]	5.0	<1.0
12	>5	>5		8.20	<1.6
2: etoposide	not made		1.03	8.64	8.4

[a] Numbers are for the fluorinated compounds. [b] SI = selectivity index, ED_{50}/ID_{50} of 2-F compounds or of 2-H compound for **2** (etoposide). [c] ID_{90} = 0.86 μM.

Table 4. Data for Antineoplastic Screening of 4β-Anilino-2-fluoro Derivatives

		percent inhib (%)			
compd	concn (μg/mL)	KB cells	CAKI-1 cells	A549 cells	MCF-7 cells
13	4	70.9	44.3	61.4	61.2
	0.4	10.1	15.3	26.2	20
	0.04	7.5	10.3	16.1	13.2
14	4	30.5	28.4	52.5	45.8
	0.4	4.2	9.5	11.9	8.2
	0.04	5	7.9	11.1	7.7
15	4	41.6	21.9	51.8	42.1
	0.4	3.8	2.6	6.5	7.3
	0.04	0	0.8	0	0.9
16	4	30.5	26.3	60.1	29.4
	0.4	0	1.3	19.6	1.4
	0.04			0	
17	4	61.5	38.0	51	48.1
	0.4	7.5	2.5	7.7	5.8
	0.04	0	0	0	0
11	4	87.4	40.5	73.1	51
	0.4	72	39.7	69.6	48
	0.04	53.3	18.1	59.4	34.2
12	4	64	39	78.1	53.8
	0.4	25.6	10.6	34.3	21.8
	0.04	0	0	0	0

Table 5. Comparison of Fluorinated and Nonfluorinated Compounds as Antineoplastic Agents

2-F compd: log GI_{50}				2-H compd: log GI_{50}			
compd	KB	CAKI	A549	compd	KB	CAKI	A549
11	−7.5	>−5	−5.4	**11-H**		−7.0	−8.1
13	−5.7	>−5	−5.7	**13-H**	−6.6	−6.5	−6.6
15	>−5	>−5	−5.4	**15-H**	−6.3	−6.8	−6.8
16	>−5	>−5	−5.4	**16-H**	−6.2	−6.9	−7.0
17	−5.4	>−5	−5.4	**17-H**	−6.1	−6.5	−6.5
2-F	not made			**2**	−6.7	−5.2	−5.2

and etoposide and 4β-anilino compounds both function as DNA topo II inhibitors.

Human cytomegalovirus (HCMV) is another type of herpesvirus that has come into recent prominence with the emergence of the AIDS pandemic. It is one of the numerous devastating opportunistic infections contracted by AIDS patients. HCMV retinitis, infection in the eyes of a patient, frequently results in blindness.[35] In non-AIDS patients, the worst effects of HCMV are in infants. Between 1:200 and 1:100 births in the United States involves congenital or maternal transmission, respectively, of HCMV to the newborn; 63% of congenitally infected infants have some form of psychomotor damage, and 59% suffer hearing loss. Also, a significant portion (47%) will meet the clinical definition of mentally retarded, and 36% of these children will also meet the definition of severely mentally retarded.[36] HCMV infection activates cellular DNA topo II synthesis, and this activation is essential for viral DNA replication. Accordingly, topo II inhibitors can exhibit good activity against HCMV. Thus, we decided to test the fluorinated series of compounds against HCMV.[37,38]

The results of the HCMV screening are shown in Table 3. Most compounds exhibited moderate activity against HCMV in the virus yield (as measured by plaque assay) and in DNA dot−dot hybridization assays. For **13**−**16**, the ID_{50} and ID_{90} values were in the range of 1 μM and 4 μM, respectively. Compounds **15** and **16** also exhibited a narrow therapeutic range. Where the data was available, comparison was made to the nonfluorinated analogues.[39] For most compounds, activity decreased slightly (1.4- to 4-fold) upon fluorination. One unusual result from this study was the complete inactivity of **11** and **12**. While 2-fluoropodophyllotoxin (**11**) showed no activity, podophyllotoxin (**1**) had an ID_{50} below 100 nM. This dramatic loss of activity is unexplained. The ED_{50} values for HEL cell growth are also shown in Table 3 along with a selectivity index. For all tested compounds, the ED_{50} was calculated against the growing HEL cell. The fluorinated compound **11** was extremely toxic. Compound **14** exhibited a selectivity index of 11, while most of the remaining derivatives showed values in the range of 3−8. Compounds **11** and **12** did not demonstrate any selectivity.

The antiviral mechanisms of action can only be speculative at this time. The HSV inhibition by podophyllotoxin and the related **9** and **11** could be related to antimicrotubule activity and consequent inhibition of viral transport, and the anti-HCMV activity of the β-anilino-4′-demethyl compounds could be related to their topo II inhibition. However, although the topo II inhibitor etoposide causes irreversible inhibition of HCMV DNA synthesis, topo II involvement has not been definitely associated.[38]

Compounds **2**−**5** all are either in clinical use or in clinical trials against various cancers; accordingly, the fluorinated compounds were examined versus several different cancer cell lines, including KB cells (nasopharyngeal carcinoma), CAKI-1 cells (renal carcinoma), A549 cells (lung cancer), and MCF-7 cells (breast cancer). Table 4 presents the screening data as the percent inhibition of growth compared to the control (untreated) cells. The GI_{50} values for the fluorinated compounds and the nonfluorinated analogues are compared in Table 5. As was seen in the antiviral screens, these compounds were moderately active against some of the cancer cell lines, but they were less active than the corresponding nonfluorinated analogues. Compound **11** exhibited the best activity against KB carcinoma with a GI_{50} of approximately 30 nM, while **13** and **17** had GI_{50} values of 2 and 4 μM, respectively. Although not as potent as the nonfluorinated compounds, the decreased cytotoxicity may portend a greatly increased therapeutic index. Further study is underway to determine exact therapeutic indices.

In conclusion, fluorination of the 2-position appears not to be a positive modification for the podophyllotoxins. The dramatic loss of activity in many of the screens is unusual and difficult to explain. The steric parameters of hydrogen and fluorine are extremely similar (van der Waals radii of 1.2 Å for hydrogen vs 1.35 Å for fluorine). Although the calculated charges do not indicate an increased likelihood for hydrolysis of the lactone, the explanation may nevertheless be electrostatic in nature. Previous pharmacophoric models of these compounds have suggested that they may interact with DNA in a nonintercalative manner.[40] Thus, because the phosphate backbone of DNA is strongly negatively charged and the fluorine atom has an increased partial negative charge (due to a through-space effect), the DNA−compound interaction may be inhibited with the fluorinated compounds. Accordingly, if such interaction with DNA is essential to the primary mechanism of action for these compounds, any decrease could result in a dramatic loss of activity. Further studies, including DNA binding and topo II inhibition assays, are underway to determine the extent and importance of the interaction with DNA and its relation to antiviral mechanism of action.

Experimental Section

All reagents were purchased from Aldrich Chemical Co. unless otherwise specified. Water used in all reactions was deionized. "Dry" solvents were distilled from an appropriate drying agent and stored over 4 Å molecular sieves until used. Oven-dried glassware was stored in a 145 °C drying oven for a minimum of 12 h prior to use. Molecular sieves were dried at 350 °C for 6 h then stored at 145 °C until used. All NMR assignments (δ, ppm) were made using a combination of 1D and 2D methods, including COSY, NOSY, HETCOR, and LR-HETCOR.

Preparation of 4′-*O*-Demethyl-2-fluoropodophyllotoxin (12). 2-Fluoropodophyllotoxin (**11**) (8.1 mmol) was dissolved in 100 mL of dry CH_2Cl_2 and cooled to 0 °C under an Ar(g) atmosphere. Trimethylsilyl iodide (3.1 equiv), as a solution in 10 mL of dry CH_2Cl_2, was added slowly over 30 min. The reaction was stirred at 0 °C for 6 h and poured into 100 mL of 1:1 water:acetone (precooled to 0 °C). $BaCO_3$ (10 mmol) was added, and the reaction mixture was heated to 40 °C for 60 min. After cooling, it was poured into 500 mL of 10% (w/v) $Na_2S_sO_3$(aq) and stirred for 60 min. The layers were separated, and the water later was extracted with 2 × 100 mL of CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude solid was dissolved in a minimum of hot acetone, and the product was precipitated with hexanes. The supernatant was then reduced by rotary evaporation and the starting material was recovered: yield = 80% (25% conversion based on recovered starting material); mp = 237 °C dec; TLC (6:4 hexanes: acetone) R_f = 0.50; ^{1}H NMR (400 MHz, acetone-d_6) 4.72 (d, 1H, H1), 3.13 (dq, 1H, H3), 5.07 (d, 1H, H4), 2.86 (br, 1H, 4-OH), 7.06 (s, 1H, H5), 6.59 (s, 1H, H8), 4.66 (dd, 1H, H11α), 4.55 (dd, 1H, H11β), 6.00 (d, 2H, H14), 6.38 (s, 2H, H2′), 3.67

(s, 6H, 3′-OCH_3); ^{13}C NMR (100 MHz, acetone-d_6) 49.88 (C1), 97.65 (C2), 41.51 (C3), 68.51 (C4), 102.31 (C5), 148.16 (C6), 149.14 (C7), 109.85 (C8), 128.25 (C9), 132.87 (C10), 68.51 (C11), 170.42 (C13), 102.32 (C14), 132.87 (C1′), 110.22 (C2′), 136.42 (C3′), 129.40 (C4′), 56.63 (C3′-OCH_3); ^{19}F NMR (376.5 MHz, acetone-d_6) −162.50 (dd, $^3J_{1H/2F} = 11.1$ Hz, $^3J_{2F/3H} = 40.8$ Hz). Anal. ($C_{21}H_{19}FO_8$) C, H.

General Preparation of 4β-Anilino-4′-*O*-demethyl-2-fluoropodophyllotoxins 13−16. In dry glassware, **12** (0.10 mmol) was dissolved in 10 mL of dry 1,2-dichloroethane and 1 mL of dry diethyl ether and cooled to 0 °C. HBr(g) was bubbled into the reaction for 20 min. Excess gas was scrubbed with a $NaHCO_3$(aq,satd) bubbler. After stirring for 2 h at 0 °C, the solvent was removed under reduced pressure to give a slightly colored oil. The oil was dissolved in 10 mL of dry THF. The substituted aniline (0.30 mmol) and $BaCO_3$ (0.40 mmol) were then added and stirred at ambient temperature for several hours. The reaction was then filtered through a pad of Celite, and the solvent was removed under reduced pressure. The crude solid was chromatographed on the FlashElute chromatography system using a 25M silica cartridge.

4β-(*p*-Fluoroanilino)-4′-*O*-demethyl-2-fluoropodophyllotoxin (13): yield = 45%; mp = 188−9 °C; ^1H NMR (400 MHz, acetone-d_6) 4.75 (d, 1H, H1), 3.39 (dq, 1H, H3), 5.24 (d, 1H, H4), 6.87 (s, 1H, H5), 6.60 (s, 1H, H8), 4.54 (dd, 1H, H11α), 4.34 (dd, 1H, H11β), 5.98 (d, 2H, H14), 6.43 (s, 2H, H2′), 3.70 (s, 6H, 3′-OCH_3), 6.94 (m, 2H, H2″), 7.49 (m, 2H, H3″); ^{19}F NMR (376.5 MHz, acetone-d_6) −128.87 (t, 1F, F4″), −162.95 (dd, 1F, F2, $^3J_{1H/2F} = 11.2$ Hz, $^3J_{2F/3H} = 41.4$ Hz); chromatography solvent: 1:1 EtOAc:hexanes. Anal. ($C_{27}H_{23}F_2NO_7$) C, H, N.

4β-(*p*-Cyanoanilino)-4′-*O*-demethyl-2-fluoropodophyllotoxin (14): yield = 68%; mp = 199−201 °C; ^1H NMR (400 MHz, acetone-d_6) 4.77 (d, 1H, H1), 3.45 (dq, 1H, H3), 5.42 (d, 1H, H4), 6.90 (s, 1H, H5), 6.62 (s, 1H, H8), 4.59 (dd, 1H, H11α), 4.25 (dd, 1H, H11β), 6.00 (d, 2H, H14), 6.43 (s, 2H, H2′), 3.70 (s, 6H, 3′-OCH_3), 6.99 (m, 2H, H2″), 7.50 (m, 2H, H3″); ^{19}F NMR (376.5 MHz, acetone-d_6) −162.87 (dd, $^3J_{1H/2F} = 14.3$ Hz, $^3J_{2F/3H} = 39.9$ Hz); chromatography solvent: 3:2 EtOAc: hexanes. Anal. ($C_{28}H_{23}FN_2O_7$) C, H, N.

4β-(*p*-Nitroanilino)-4′-*O*-demethyl-2-fluoropodophyllotoxin (15): yield = 70%; mp = 192−3 °C; ^1H NMR (400 MHz, acetone-d_6) 4.77 (d, 1H, H1), 3.50 (dq, 1H, H3), 5.54 (d, 1H, H4), 6.92 (s, 1H, H5), 6.64 (s, 1H, H8), 4.66 (dd, 1H, H11α), 4.25 (dd, 1H, H11β), 6.01 (d, 2H, H14), 6.44 (s, 2H, H2′), 3.69 (s, 6H, 3′-OCH_3), 7.90 (m, 2H, H2″), 8.44 (m, 2H, H3″); ^{19}F NMR (376.5 MHz, acetone-d_6) −162.68 (dd, $^3J_{1H/2F} = 9.4$ Hz, $^3J_{2F/3H} = 39.5$ Hz); chromatography solvent: 1:1 EtOAc: hexanes. Anal. ($C_{27}H_{23}FN_2O_9$) C, H, N.

4β-(3″,4″-(Ethylenedioxy)anilino)-4′-*O*-demethyl-2-fluoropodophyllotoxin (16): yield = 85%; mp = 225−7 °C; ^1H NMR (400 MHz, acetone-d_6) 4.77 (d, 1H, H1), 3.44 (dq, 1H, H3), 5.70 (d, 1H, H4), 6.92 (s, 1H, H5), 6.62 (s, 1H, H8), 4.59 (dd, 1H, H11α), 4.27 (dd, 1H, H11β), 6.00 (d, 2H, H14), 6.44 (s, 2H, H2′), 3.70 (s, 6H, 3′-OCH_3), 6.90 (d, 1H, H2″), 7.82 (d, 1H, H2″), 7.82 (d, 1H, H3″), 4.24 (m, 2H, H−OCCO); ^{19}F NMR (376.5 MHz, acetone-d_6) −128.87 (t, 1F, F4″), −162.80 (dd, 1F, F2, $^3J_{1H/2F} = 13.2$ Hz, $^3J_{2F/3H} = 43.3$ Hz); chromatography solvent: 1:1 EtOAc:hexanes. Anal. ($C_{29}H_{26}FNO_9$) C, H, N.

Preparation of 4β-(*p*-Aminoanilino)-4′-*O*-demethyl-2-fluoropodophyllotoxin (17). In a Parr bottle, **15** (0.10 mmol) was dissolved in 25 mL of EtOAc and 50 mg of 10% Pd/C was added. The reaction was placed on a hydrogenator with 50 psi of H_2(g) and shaken for 12 h. The reaction was removed and filtered through Celite to give a clear (nonyellow) solution. TLC gave only one spot and no starting material. The solvent was removed under reduced pressure to give an off white solid: yield = 95%; mp = 180 °C dec; ^1H NMR (400 MHz, acetone-d_6) 4.81 (d, 1H, H1), 3.52 (dq, 1H, H3), 5.50 (d, 1H, H4), 6.96 (s, 1H, H5), 6.71 (s, 1H, H8), 4.64 (dd, 1H, H11α), 4.27 (dd, 1H, H11β), 5.98 (d, 2H, H14), 6.42 (s, 2H, H2′), 3.68 (s, 6H, 3′-OCH_3), 7.70 (m, 2H, H2″), 8.45 (m, 2H, H3″); ^{19}F NMR (376.5 MHz, acetone-d_6) −162.97 (dd, $^3J_{1H/2F} = 11.6$ Hz, $^3J_{2F/3H} = 40.2$ Hz). Anal. ($C_{27}H_{25}FN_2O_7$) C, H, N.

Herpesvirus. 1. Cell Growth Inhibition Assay.[32] Vero cells (African green monkey kidney cells) were cultured in RPMI-1640 with 100 μg/mL of kanamycin and 5% (v/v) of calf serum in a humidified atmosphere containing 5% CO_2. Cultures were seeded at 50 000 cells/mL in 96-microtiter-well format with various concentrations of the test compounds. Drug exposure was for 2 days and cell numbers were determined by the sulforhodamine B (SRB) staining method. The ED_{25} value, the drug concentration that reduced absorbency by 25%, was interpolated from dose−response data. The MIC concentration is the minimum value from the dilution series that gave detectable cell growth inhibition. Each test was performed in triplicate, and absorbency readings varied by no more that 5%. ED_{25} values determined in independent tests varied by no more than 30%.

2. Plaque Elimination Assay.[32] Antiviral activity was evaluated in pre-formed Vero monolayer cultures using KOS (HSV-1) and 186 (HSV-2) viral strains. Confluent Vero cultures were inoculated with approximately 100 PFU (plaque forming units) of virus, and after 30 min, the inoculum was replaced with 1 mL of medium supplemented with 0.5% (v/v) calf serum, 1% (v/v) carboxymethylcellulose and drugs at various concentrations. The cells were stained and fixed after 2 days with 0.8% (w/v) crystal violet in 50% ethanol and macroscopic plaques were scored. Determinations of drug susceptibility were repeated at least twice on separate occasions and reproducible results were obtained. The ED_{100} concentration is the dilution tested that completely eliminated macroscopic plaque formation without over toxicity to cell monolayers based on staining intensity and microscopic examination prior to staining. The MIC concentration value is the minimum value from the dilution series that gave antiviral effect (i.e. reduction in plaque size and/or number relative to infected controls).

Cytomegalovirus. 1. Titer Reduction Assay.[37] Extracellular virus yield was measured by plaque formation assay in human embryonic lung (HEL) fibroblasts. In brief, HEL cells were grown to confluency in 24-well culture plates. Cells were rinsed once with MEM, then infected with Towne strain CMV at a multiplicity of 1−2 PFU/cell in the presence or absence of various concentrations of drug. Media with appropriate concentration of drug were changed on the day 3 post-infection. On day 6 post-infection, the culture supernatant was removed and used to perform a standard CMV plaque assay in HEL cells. 10-fold serial dilution and 1% methylcellulose (in 4% fetal calf−MEM medium) overlayer were employed. The plaque number was counted 2 weeks after infection under an inverted microscope.

2. DNA−DNA Dot Hybridization Assay.[38] Viral DNA synthesis was monitored by DNA−DNA dot hybridization using nick-translated ^{32}P-labeled CMV DNA as the probe. Confluent HEL fibroblasts (human embryonic lung, HEL 229, ATCC 137-CLL), on 24-well culture plates, were infected with Towne strain CMV at the multiplicity of 1−2 FPU/cell. After 2 h of absorption, MEM medium (supplemented with 4% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin) and drugs at various concentrations were added to the CMV-infected cultures. At various times after infection, CMV-infected and mock-infected HEL cells, with or without drug treatment, were lysed with 0.2 mL of lysis buffer solution [0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA, 1% sodium dodecyl sulfate (SDS), and 0.1 M $CaCl_2$], and digested with proteinase K at 100 μg/mL for 1 h at 37 °C. The lysate was extracted with phenol−chloroform (1:1) once, chloroform once, and then precipitated with 2.5 vol of alcohol at −20 °C overnight. The nucleic acid precipitate was dissolved in 0.2 mL of TE buffer (0.01 M Tris-HCl, pH 7.4, and 0.001 M EDTA). For dot hybridization, 0.05 mL of the infected or mock-infected DNA solution from each experimental sample was first denatured in 0.5 M NaOH with 1.5 M NaCl for 1 h at room temperature and then neutralized on ice with 1.1 N HCl in 0.2 M Tris to a final pH around 7.4. The mixture was then adjusted to 6 × SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate), and single-stranded DNA was immobilized on nitrocellulose membrane using Minifold apparatus (Schleicher and Schuell,

Keene, NH 03431). The ^{32}P-labeled nick-translated Towne strain of HCMV DNA (specific activity: 5×10^7 to 1×10^8 cpm/μg) at a level of 1×10^6/mL of hybridization solution was used. Hybridization was carried out at 66 °C for 18 h. Kodak RP/R2 X-ray film was used for radioautography to trace the radiolabeled ^{32}P DNA hybridized. After autoradiography, individual dots from samples on the hybridized membrane were cut for measuring the radioactivity by liquid scintillation counter. All assays were performed in duplicate with reproducible results generated for each compound.

3. HEL Cytotoxicity Assay.[38] HEL cells were cultured in MEM with 10% (v/v) FBS, 100 units and 100 μg/mL each of penicillin and streptomycin, respectively, in a humidified atmosphere containing 6% CO_2. Cultures were seeded at 50 000 cells/mL in 24-well plates (1 mL/well) with various concentrations of the test compounds. Drug exposure was followed for 15 days, and cell viability and cell numbers were determined by the trypan blue staining method. The ED_{50} value, the drug concentration that reduced cell growth as compared to nontreated cells by 50%, was interpolated from dose−response data. Each test was performed in triplicate, and readings varied by no more that 5%. ED_{50} values determined in independent tests varied by no more than 30%.

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References

(1) For part 206, see: Lee, K. H. Antitumor Agents. 206. Anti-AIDS Agents. 39. Molecular Modifications of Lead Compounds to Develop Potent Antitumor and Anti-HIV Agents. Abstracts of UNESCO Regional Symposium on Chemistry of Medicinal Plants, Kumming, China, 2000; pp 14−26.

(2) Stähelin, H. Activity of a New Glycosidic Lignan Derivative (VP-16-213) Related to Podophyllotoxin in Experimental Tumors. *Eur. J. Cancer* **1973**, *9*, 215−221.

(3) Stähelin, H. 4′-Demethylepipodophyllotoxin Thenylidene Glucoside (VM 26), a Podophyllum Compound with a New Mechanism of Action. *Eur. J. Cancer* **1970**, *6*, 303−311.

(4) The 'epi' prefix attached to the podophyllotoxin name indicates that the stereochemistry in the C-4 position is β (up relative to the drawn structure and *trans* relative to the E-ring). Without this or other notation, the structure is α (down at the C-4 position relative to the drawn structure and *cis* to the E-ring).

(5) Rassmann, I.; Schrodel, H.; Schilling, T.; Zucchetti, M.; Kaeser-Frohlich, A.; Rastetter, J.; Hanauske, A. R. Clinical and Pharmacokinetic Phase I Trial of Oral Dimethylaminoetoposide (NK611) Administered for 21 Days Every 35 Days. *Invest. New Drugs* **1996**, *14*, 379−86.

(6) Zhu, X. K.; Guan, J.; Tachibana, Y.; Bastow, K. F.; Cho, S. J.; Cheng, H. W.; Cheng, Y. C.; Gurwith, M.; Lee, K. H. Antitumor Agents 194. Synthesis and Biological Evaluations of 4β-Mono-, Di-, and Trisubstituted Aniline-4′-*O*-demethyl-podophyllotoxin and Related Compounds with Improved Pharmacological Profiles. *J. Med. Chem.* **1999**, *42*, 2441−2446 and literature cited therein.

(7) Budman, D. R.; Igwemezie, L. N.; Kaul, S.; Behr, J.; Lichtman, S.; Schulman, P.; Vinciguerra, V.; Allen, S. L.; Kolitz, J.; Hock, K.; O'Neil, K.; Schacter, L.; Barbhaiya, R. H. Phase I Evaluation of a Water-Soluble Etoposide Prodrug, Etoposide Phosphate, Given as a 5-Minute Infusion on Days 1, 3, and 5 in Patients with Solid Tumors. *J. Clin. Oncol.* **1994**, *12*, 1902−1909.

(8) Engelborghs, Y.; Fitzgerald, T. J. A Fluorescence Stopped Flow Study of the Competition and Displacement Kinetics of Podophyllotoxin and the Colchicine Analogue 2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone on Tubulin. *J. Biol. Chem.* **1987**, *262*, 5204−5209.

(9) Yanagida, M.; Sternglanz, R. In *DNA Topology and its Biological Implications*; Cozzarelli, N. R., Wang, J. C., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1990; pp 243−263.

(10) Huang, T. S.; Shu, C. H.; Yang, W. K.; Whang-Peng, J. Activation of CDC25 Phosphatase and CDC2 Kinase Involved in GL331-Induced Apoptosis. *Cancer Res.* **1997**, *57*, 2974−2978.

(11) Shimizu, T.; O'Connor, P. M.; Kohn, K. W.; Pommier, Y. Unscheduled Activation of Cyclin B1/CDC2 Kinase in Human Promyelocytic Leukemia Cell Line HL60 Cells Undergoing Apoptosis Induced by DNA Damage. *Cancer Res.* **1995**, *55*, 228−231.

(12) Van Maanen, J. M.; Retel, J.; de Vries, J.; Pinedo, H. M. Mechanism of Action of Antitumor Drug Etoposide: a Review. *J. Natl. Cancer Inst.* **1988**, *80*, 1526−1533.

(13) Sakurai, H.; Miki, T.; Imakura, Y.; Shibuya, M.; Lee, K. H. Metal and Photoinduced Cleavage of DNA by Podophyllotoxin, Etoposide, and Their Related Compounds. *Mol. Pharmacol.* **1991**, *40*, 965−973.

(14) Saulnier, M. G.; Vyas, D. M.; Langley, D. R.; Doyle, T. W.; Rose, W. C.; Crosswell, A. R.; Long, B. H. E-Ring Desoxy Analogues of Etoposide. *J. Med. Chem.* **1989**, *32*, 1418−1420.

(15) Cragg, G.; Suffness, M. Metabolism of Plant-Derived Anticancer Agents. *Pharmacol. Ther.* **1988**, *37*, 425−461 and references therein.

(16) Hande, K. R.; Combs, G. L.; Cotham, R.; Guengerich, F. P.; Branch, R. A. Biliary Clearance and Hepatic Metabolism of Etoposide (VP-16). *Proc. Am. Soc. Clin. Oncol.* **1985**, *4*, 34−50.

(17) Ho, D. H.; Kanellopoulow, K. A.; Yap, Y. H.; Casimir, M.; Savaraj, N.; Issell, S. B.; Benjamin, R. S.; Bodey, E. P. Clinical Pharmacology of Etoposide by Radioimmunoassay. *Proc. Am. Assoc. Cancer Res.* **1983**, *24*, 519−526.

(18) Gensler, W. J.; Gatsonis, C. D. The Podophyllotoxin − Picropodophyllin Equilibrium. *J. Org. Chem.* **1966**, *31*, 3224−3227.

(19) Glinski, M. B.; Freed, J. C.; Durst, T. Preparation of 2-Substituted Podophyllotoxin Derivatives. *J. Org. Chem.* **1987**, *52*, 2749−2753.

(20) Pearce, H. L.; Bach, N. J.; Cramer, T. L. Synthesis of 2-Azapodophyllotoxin. *Tetrahedron Lett.* **1989**, *30*, 907−910.

(21) Tomioka, K.; Kubota, Y.; Koga, K. Synthesis and Antitumor Activity of Podophyllotoxin Aza-Analogues. *Tetrahedron Lett.* **1989**, *30*, 2953−2954.

(22) Gensler, W. J.; Murthy, C. D.; Trammell, M. H. Nonenolizable Podophyllotoxin Derivatives. *J. Med. Chem.* **1977**, *20*, 635−644.

(23) Jardine, I.; Strife, R. J.; Kozlowski, J. Synthesis, 470-MHz ^{1}H NMR Spectra, and Activity of Delactonized Derivatives of the Anticancer Drug Etoposide. *J. Med. Chem.* **1982**, *25*, 1077−1081.

(24) Vaitkevicius, V. K.; Reed, M. L. Clinical Studies with Podophyllum Compounds SPI-77 (NSC-72274) and SPG-827 (NSC-42076). *Cancer Chemother. Rep.* **1966**, *50*, 565−571.

(25) VanVliet, D. S.; Lee, K. H. A High Yield Preparation of 2-Fluoropodophyllotoxin. *Tetrahedron Lett.* **1999**, *40*, 2259−2262..

(26) Corey, E. J.; Venkateswarlu, A. A New Silyl Protecting Group. *J. Am. Chem. Soc.* **1972**, *94*, 6190−6191.

(27) Davis, F.; Han, W. *N*-Fluoro-*O*-Benzenedisulfonimide: A Useful New Fluorinating Reagent. *Tetrahedron Lett.* **1991**, *32*, 1631−1634.

(28) Differding, E.; Ofner, H. *N*-Fluorobenzenesulfonimide: A Practical Reagent for Electrophilic Fluorinations. *Synlett* **1991**, 187−189.

(29) This test was done using TLC developed with $FeCl_3$ (2.7% w/v in 2 N HCl) purchased from Sigma.

(30) Daley, L.; Meresse, P.; Bertounesque, E.; Monneret, C. A One-Pot, Efficient Synthesis of the Potent Cytotoxic Podophyllotoxin Derivative NPF. *Tetrahedron Lett.* **1997**, *38*, 2673−2676.

(31) Zhang, Y.; Lee, K. H. Recent Progress in the Development of Novel Antitumor Etoposide Analogues. *Chinese Pharm. J.* **1994**, *46*, 319−369 and references therein.

(32) Bastow, K. F.; Derse, D. D.; Cheng, Y. C. Susceptibility of Phosphonoformic Acid-Resistant Herpes Simplex Virus Variants to Arabinosylnucleosides and Aphidicolin. *Antimicrob. Agents Chemother.* **1983**, *23*, 914−917.

(33) Lee, K. H. Unpublished results.

(34) Akanitapichat, P.; Lowden, C. T.; Bastow, K. F. 1,3-Dihydroxyacridone Derivatives as Inhibitors of Herpes Virus Replication. *Antiviral Res.* **2000**, *45*, 123−134.

(35) Hoover, D. R.; Saah, A. J.; Bacellar, H.; Phair, J.; Detels, R.; Anderson, R. Clinical Manifestations of AIDS in the Era of Pneumocystis Prophylaxis. Multicenter AIDS Cohort Study. *N. Engl. J. Med.* **1993**, *329*, 1922−1926.

(36) Boppana, S. B.; Fowler, K. B.; Vaid, Y.; Hedlund, G.; Satgno, S.; Britt, W. J.; Pass, R. F. Neuroradiographic Findings in the Newborn Period and Long-Term Outcome in Children with Symptomatic Congenital Cytomegalovirus Infection. *Pediatrics* **1997**, *99*, 409−414.

(37) Johnson, R. A.; Huong, S. M.; Huang, E. S. Inhibitory Effect of 4-(4-Fluorophenyl-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole on HCMV DNA Replication. *Antiviral Res.* **1999**, *41*, 101−111.

(38) Huang, E. S.; Benson, J. D.; Huong, S. M.; Wilson, B.; van der Horst, C. Irreversible Inhibition of Human Cytomegalovirus Replication by Topoisomerase II Inhibitor, Etoposide: a New Strategy for the Treatment of Human Cytomegalovirus Infection. *Antiviral Res.* **1992**, *17*, 17−32.

(39) Huang, E. S.; Lee, K. H. Unpublished results.

(40) Cho, S. J.; Tropsha, A.; Suffness, M.; Cheng, Y. C.; Lee, K. H. Antitumor Agents. 163. Three-Dimensional Quantitative Structure−Activity Relationship Study of 4′-*O*-Demethylepipodophyllotoxin Analogues Using the Modified CoMFA/q^2-GRS Approach. *J. Med. Chem.* **1996**, *39*, 1383−1395.

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