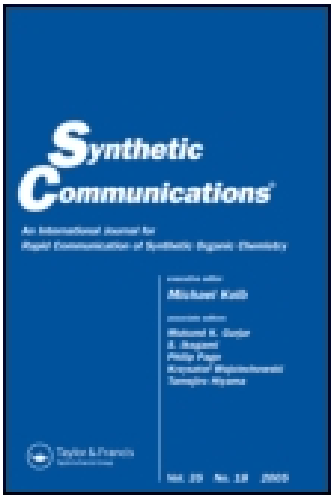

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Synthesis of Novel Spin-Labeled Derivatives of Podophyllotoxin as Potential Antineoplastic Agents

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Abstract: Five novel nitroxyl spin-labeled ester derivatives of podophyllotoxin have been prepared by reacting the corresponding N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxy-carbonyl) amino acids with the hydroxy group of podophyllotoxin in the presence of dimethylaminopyridine and N,N-dicyclohexylcarbodiimide and evaluated as potential antitumor agents. All of the target compounds showed more significant cytotoxicity against P-388 murine leukemia and A-549 human lung carcinoma in vitro than etoposide.

Keywords: Antineoplastic properties, podophyllotoxin derivatives, spin-labeled

INTRODUCTION

Podophyllotoxin (**1**) and its many related derivatives are well known to have pronounced antineoplastic and antiviral properties.[1] Its semisynthetic analogues of etoposide (**2**) and teniposide (**3**) are currently used in chemotherapy for various types of cancer, including small-cell lung cancer, acute leukemia, lymphoma, testicular carcinoma, and Kaposi's sarcoma.[2–4] Interestingly, these semisynthetic derivatives and the parent compound, podophyllotoxin, showed different mechanisms of action. Podophyllotoxin inhibits tubulin polymerization through its interaction at the colchicines binding site,[5] whereas etoposide and congeners involve the inhibition of topoisomerase II–mediated DNA ligation by reversible cleavable complex

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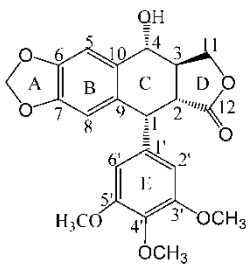
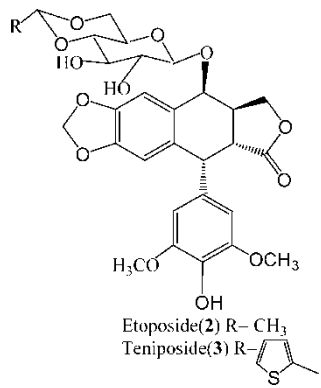
stabilization resulting in tumor-cell death and its corresponding ortho-quinone via oxidative demethylation.[6] However, because of the typical adverse effects common to most antineoplastics, such as anemia, hair loss, and severe gastrointestinal disturbances, the clinical application of them has been limited to a certain extent. In this regard, it is necessary to continuously research and synthesize podophyllotoxin and etoposide analogues that encompass a broad spectrum of antineoplastic activities and overcome several clinical limitations.

Early efforts by clinicians and research scientists to reduce the side effects of podophyllotoxin and maintain or enhance activity were directed along the lines of ester synthesis,[7,8] especially because these derivatives showed strong activity against P-388 lymphocytic leukemia inoculated into mice. Although several of these esters showed significant activity, these derivatives themselves have side effects because of general toxicity. In previous studies we found that the introduction of a stable nitroxyl radical into the molecule of podophyllotoxin could result in new compounds, which have significant antitumor activity with marked decrease in toxicity when compared with the parent compounds.[9–12] A series of spin-labeled derivatives of thio-TEPA,[13] nitrosourea,[14] rubomycin,[15] and 6-mercaptopurine[16] were also reported to have pharmacological properties superior to their parent compounds. Recently, it has been found that the nitroxyl radicals can normalize the level of the oxidized form of P-450 cytochrome, which has been brought down by the injection of cytostatic agents of lethal doses, and the nitroxyl moiety serves as a transport vehicle through cell membranes.[17] Therefore, nitroxyl radicals can be considered as biological response modifiers.

Based on this, as well as the fact that ‘L-amino acids are actively transplanted into mammalian tissue, have good water solubility, and are often used as carrier vehicles for some drugs, we designed five novel spin-labeled derivatives with the hope that C-4-OH group esterified by L-amino acids containing a nitroxyl radical moiety would simultaneously circumvent the several limitations and develop superior pharmacological profiles. Herein, we report the synthesis, biological activity, and structures of five novel spin-labeled compounds.



Podophyllotoxin(**1**)



Etoposide(**2**) R– CH_3
Teniposide(**3**) R–

CHEMISTRY

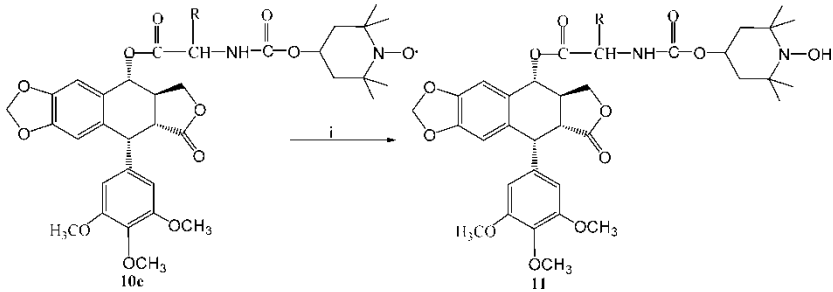
Podophyllotoxin (**1**) was isolated from a Chinese medicinal herb *Podophyllum emodi* Wall var Chinesis Sprague and served as the starting material for the preparations of all the derivatives.

4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**5**) has been prepared by means of catalytic oxidation of 4-hydroxy-2,2,6,6-tetramethylpiperidine (**4**) with sodium tungstate-hydrogen peroxide–EDTA.[18] The reaction of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**5**) with N,N′-carbonyldiimidazole procceds to give the intermediate N-(1-oxyl-2,2,6,6-tetramethylpiperidinyloxycarbonyl)-imidazole (**6**) by the modified method that Staab described for the synthesis of N-t-butoxycarbonylimidazole.[19] The intermediate (**6**), without isolation, was further reacted with *p*-toluenesulfonic acid monohydrate to give its higher reactive tosylate (**7**). This salt is so reactive that it is instantaneously converted into compound **8** when dissolved in an aqueous solution of sodium azide. Compounds **9a**–**9e** can be obtained in good yield by reaction of the alkoxycarbonyl azide (**8**) with free amino acids.[20] Synthesis of the desired compounds **10a**–**10e** was accompanished by reaction of compounds **9a**–**9e** with podophyllotoxin in the presence of N,N-dicyclohexylcarbodiimide (DCC) and catalytic amount of dimethylaminopyridine (DMAP) to form an ester linkage under an atmosphere of nitrogen. The rate of reduction of the representative compound **10e** in $CDCl_3$ into the corresponding N-hydroxy amine compound **11** using phenylhydrazine was monitored visually by the disappearance of the characteristic red-orange color of the nitroxyl derivatives (Scheme 1).

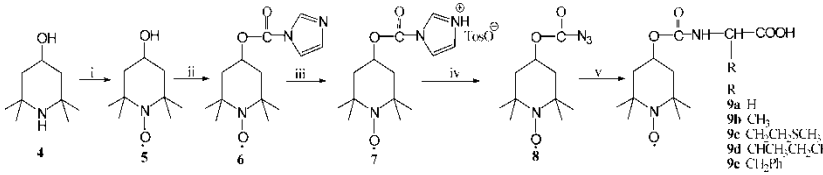
The synthetic methodology for the preparation of spin-labeled podophyllotoxin compounds **10a**–**10e** is depicted in Schemes 2 and 3.

BIOLOGICAL EVALUATION

Cytotoxicities of the target compounds **10a**–**10e** against cell cultures of P-388 murine leukemia and A-549 human lung carcinoma were tested in vitro



Scheme 1. Reagents and conditions: i, phenylhydrazine/$CDCl_3$, 25 °C, 10 min.

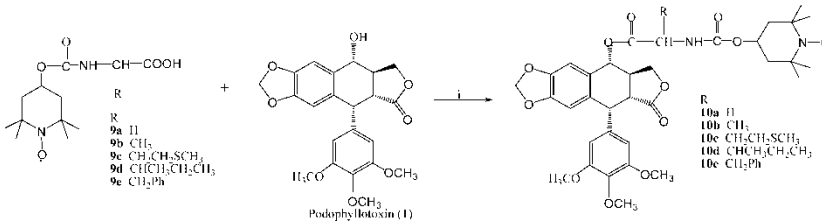


Scheme 2. Reagents and conditions: i, Na_2WO_4/H_2O_2/EDTA; ii, N,N′-carbonyldimidazole/THF, stir; iii, *p*-toluenesulfonic acid monohydrate; iv, NaN_3/water, stir; v, amino acids/MgO, stir, 24 h.

following the methods described by Bergeron.[21] The prototypical inhibitor etoposide was included as a reference standard; the results of these assays were used to obtain the corresponding inhibition rates shown in Table 1.

RESULTS AND DISCUSSION

The reaction of the hydroxy group at C-4 of podophyllotoxin with the various N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl) amino acids gave the corresponding podophyllotoxin ester derivatives in excellent yields. Owing to the paramagnetic nature of the nitroxyl spin labels, however, it is difficult to conveniently get the valuable structural information on these molecules using ^{1}H NMR spectroscopy. It occurred to us that essentially the same structural information could be obtained from the ^{1}H NMR spectra of the corresponding N-hydroxy amines. In addition, because all N-hydroxypiperidines are air- and moisture-sensitive molecules, these compounds also are air-oxidizable back to the parent nitroxyl free radical, especially in solution. Therefore, it is essential that all work with these compounds in solution be performed as rapidly as possible. To assign the configuration at C-4 position for compounds **10a**–**10e**, we applied the in situ reduction of the representive compound **10e** in $CDCl_3$ with a solution of phenylhydrazine in $CDCl_3$, which led smoothly to the corresponding N-hydroxy amine **11**,[22] in the ^{1}H NMR spectrum of the corresponding N-hydroxy amine **11**, The 4-H proton (of carbon forming the ester linkage) is found at δ_H 5.87



Scheme 3. Reagents and conditions: i, DCC/DMAP, stir, 2 h.

Table 1. Cytotoxicity activity of **10a**–**10e** in vitro against P-388 murine leukemia and A-549 human lung carcinoma

Compound	Concentration (mol/L)	Inhibition rate (%) P-388[a]	A-549[b]
10a	10^{-4}	96.3	87.3
	10^{-5}	91.0	87.9
	10^{-6}	89.1	82.8
	10^{-7}	87.8	80.1
	10^{-8}	77.5	75.5
10b	10^{-4}	99.9	94.7
	10^{-5}	89.2	86.1
	10^{-6}	88.3	83.5
	10^{-7}	81.1	81.6
	10^{-8}	70.8	50.6
10c	10^{-4}	94.9	89.5
	10^{-5}	88.8	83.5
	10^{-6}	86.3	83.3
	10^{-7}	73.8	73.2
	10^{-8}	43.0	45.9
10d	10^{-4}	95.8	87.0
	10^{-5}	94.3	83.6
	10^{-6}	73.9	72.8
	10^{-7}	14.0	39.0
	10^{-8}	0	10.7
10e	10^{-4}	93.5	87.9
	10^{-5}	92.6	87.0
	10^{-6}	80.7	81.9
	10^{-7}	68.6	79.9
	10^{-8}	52.3	60.0
Etoposide (**2**)	10^{-4}	99.2	97.5
	10^{-5}	70.6	50.7
	10^{-6}	25.5	45.0
	10^{-7}	6.0	0
	10^{-8}	1.8	0

[a]MTT methods, drug exposure was for 72 h.
[b]SRB methods, drug exposure was for 72 h.

(1H, d, $J_{3,4} = 9.2$ Hz, 4-H). The data is in agreement with those previously reported for C-4α-substituted-podophyllotoxin derivatives[23,24]. The assignment of the configuration at the C-4 position for compound **11** was based on its $J_{3,4}$ coupling constants ($J_{3,4} = 9.2$ Hz). The C-4α-substituted compounds have a $J_{3,4} \geq 8.5$ Hz as H-3 is *trans* to H-4, whereas C-4β-

substituted compounds have a $J_{3,4} < 4.5$ Hz because of the *cis* relationship between H-3 and H-4.[25] Moreover, lack of C-4β isomers may be explained through the reaction mechanism of the help of DCC in the presence of a catalytic amount of DMAP to form ester linkage reaction;[25] α-stereoselectivity of C-4 is retained as α-bond between C-4, and the hydroxyl group does not get involved in the chemical reaction. On the basis of these facts, we elucidated the configuration at C-4 position in the series. We could verify that coupling reaction between compounds **9a**–**9e** and podophyllotoxin are stereospecific in nature. Moreover, the high-resolution mass-spectral results are full agreement with the calculated mass of these derivatives. Cytotoxicities of the target compounds **10a**–**10e** against cell cultures of P-388 murine leukemia and A-549 human lung carcinoma were tested in vitro. As illustrated in Table 1, compounds **10a**–**10e** are more active than etoposide; compound **10a** is the most potent against P-388 and A-549 cells. Examination of the different amino acid linkages and the resulting activity in the inhibition of P-388 murine leukemia in vitro reveals the following order of activity: L-glycine > L-alanine > L-phenylalanine > L-methionine > L-isoleucine, whereas the resulting activity in the inhibition of A-549 human lung carcinoma in vitro reveals the following order of activity: L-glycine > L-phenylalanine > L-alanine > L-methionine > L-isoleucine. These results show that the structures of L-amino acids have potential effects on the bioactivity of these compounds. Hence, a systemic, predictable correlation could be made between the nature of amino acids and anticancer activities. In addition, the results also indicated that the strategy of introducing a stable nitroxyl radical into the molecule of podophyllotoxin with L-amino acids may be successful and they probably have synergistic action to tumor cell lines. Further biological evaluation is in progress to better define the antitumor activity of these compounds.

EXPERIMENTAL

All melting points were taken on a Kofler melting-point apparatus and are uncorrected. IR spectra were obtained on NIC-5DX spectra photometer. ^{1}H NMR spectra were obtained by using a Bruker AM400. All chemical shifts are reported in ppm from TMS. Mass-spectral analysis was taken on a ZAB-HS and Bruker Daltonics APEXII49e instrument. Optical rotations were determined on Perkin-Elmer model 341 spectropolarimeter. ESR spectra were obtained with a Bruker ER-200D-SRC X-band spectrometer.

Natural Product Origin

Podophyllotoxin (**1**) was isolated from the ethanol extract of the Chinese medicinal herb *Podophyllum emodi* Wall var Chinesis Sprague via column

chromatography and eluting with a $CHCl_3$–MeOH (20 : 1) mobile phase. Fractions containing the product were evaporated in vacuo and the podophyllotoxin recrystallized from C_6H_6. The resulting material was of >97% purity, which was confirmed by direct comparison with an authentic sample and spectral data reported previously.[26]

Synthesis of N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxy carbonyl)-imidazolium Tosylate (7)

A solution of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**5**, 1.72 g, 0.01 mol) in dry ether (10 mL) is added dropwise to a stirred solution of N,N′-carbonyldimidazole (1.62 g, 0.01 mol) in dry tetrahydrofuran (10 mL) at room temperature. After the addition is complete, the solution is stirred for 3 h at room temperature and then extracted with water (20 mL) and the saturated sodium chloride solution. The organic phase is dried with sodium sulfate and evaporated in vacuo, giving the intermediate N-(1-oxyl-2,2,6,6-tetramethylpiperidinyloxycarbonyl) – imidazole (**6**); then to a solution of N-(1-oxyl-2,2,6,6-tetramethylpiperidinyloxycarbonyl)-imidazole (**6**, 2.66 g, 0.01 mol) in dry acetone (10 mL) is added a solution of *p*-toluenesulfonic acid monohydrate (1.90 g, 0.01 mol) in acetone (10 mL), and stirred for 5 min. Crystallization is completed by adding ether, and the product isolated by suction; yield: 3.96 g (90%).

Synthesis of 1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl Azide (8)

N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-imidazolium tosylate (**7**, 2.19 g, 0.005 mol) is added at once to a stirred solution of sodium azide (0.65 g, 0.01 mol) in water (10 mL) at room temperature. The mixture is stirred for 30 min and then extracted with hexane (20 mL). The extract is dried with sodium sulfate and evaporated in vacuo and cooled to −20°C. The product is isolated as red crystals by suction; yield: 1.02 (91%). Spectral data for **8** are identical to those reported by Hankovszky et al.[20]

General Procedure for Synthesis of N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbon yl) Amino Acids (9a–9e)

A solution of 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl azide (**8**, 2.41 g, 0.01 mol) in dioxan (20 mL) is added to a stirred suspension of the amino acids (0.01 mol) and magnesium oxide (0.8 g, 0.02 mol) in water (10 mL). The mixture is stirred for 24 h at 35–40 °C, diluted with water

(30 mL), and extracted with ethyl acetate. The aqueous phase is acidified with 10% hydrochloric acid at 0°C and extracted with ethyl acetate; the organic phase is dried with sodium sulfate and evaporated. The residue is crystallized from ether, giving N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl) amino acids (**9a**–**9e**). Spectral data for **9a**–**9e** are identical to the reported by Hankovszky et al.[20]

General Procedure for Synthesis of Target Compounds 10a–10e

A mixture of N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl) amino acids (0.001 mol), podophyllotoxin (0.414 g, 0.001 mol), and dimethylaminopyridine (DMAP, 0.1 g) was stirred in dry dichloromethane (10 mL) for 5 min at room temperature under nitrogen. N,N-dicyclohexylcarbodimide (DCC, 0.21 g, 0.001 mol) was added and the reaction mixture was stirred for 2 h. The reaction mixture was filtered, and the filterate was evaporated. The residue was separated by flask-column chromatography (gradient elution with mixtures of dichloromethane–acetone) on silica gel and monitored by TLC. Synthesized target compounds **10a**–**10e** were characterized by mp, ESR, IR, MS, and HRMS spectral analyses.

Compound **10a:** Yield: 92%; mp 135–137°C; $[\alpha]_D^{20}$ −75 (c = 0.5, CH_2Cl_2); IR (KBr) (cm^{-1}): 3373 (NH), 1780 (lactone), 1723 (NHCO), 1485, 1507, 1589 (aromatic C=C), 930 (OCH_2O), 1125, 1179, 1240 (C–O), 1371 ($NO^{\cdot}$); ESR: $g_0 = 2.0058$, $\Delta H_{pp} = 35.309$Gs, $A_N = 15.81$ Gs (triplet peak in 1×10^{-4}M, CH_2Cl_2); MS(FAB) m/z(int.%): 669 (M^+, 38), 397(100); HRMS: m/z calcd. for $C_{34}H_{41}N_2O_{12}$: 671.2811$[M + 2H]^+$; Found: 671.2802 $[M + 2H]^+$.

Compound **10b:** Yield: 90%; mp 138–140°C; $[\alpha]_D^{20}$ −68 (c = 0.5, CH_2Cl_2); IR (KBr) (cm^{-1}): 3344 (NH), 1781 (lactone), 1716 (NHCO), 1485, 1507, 1589 (aromatic C=C), 930 (OCH_2O), 1126, 1175, 1239 (C–O), 1365 ($NO^{\cdot}$); ESR: $g_0 = 2.0058$, $\Delta H_{pp} = 44.268$ Gs, $A_N = 15.81$ Gs (triplet peak in 1×10^{-4} M, CH_2Cl_2); MS (FAB) m/z (int.%): 683 (M^+, 30), 397 (100); HRMS: m/z calcd. for $C_{35}H_{43}N_2O_{12}$: 685.2967 $[M + 2H]^+$; Found: 685.2962 $[M + 2H]^+$.

Compound **10c:** Yield: 91%; mp 115–117°C; $[\alpha]_D^{20}$ −65 (c = 0.5, CH_2Cl_2); IR (KBr) (cm^{-1}): 3337 (NH), 1781 (lactone), 1719 (NHCO),1485, 1507, 1589 (aromatic C=C), 930 (OCH_2O), 1126, 1175, 1239 (C–O), 1365 ($NO^{\cdot}$); ESR: $g_0 = 2.0058$, $\Delta H_{pp} = 34.255$Gs, $A_N = 15.81$ Gs (triplet peak in 1×10^{-4} M, CH_2Cl_2); MS (FAB) m/z (int.%): 743 (M^+, 68), 397 (100); HRMS: m/z calcd. for $C_{37}H_{47}N_2O_{12}S$: 745.3001 $[M + 2H]^+$; found: 745.3001 $[M + 2H]^+$.

Compound **10d:** Yield: 83%; mp 117–118°C; $[\alpha]_D^{20}$ −58 (c = 0.5, CH_2Cl_2); IR (KBr) (cm^{-1}): 3349 (NH), 1782 (lactone), 1718 (NHCO),1485, 1507, 1589 (aromatic C=C), 930 (OCH_2O), 1126, 1178, 1239 (C–O), 1366 ($NO^{\cdot}$); ESR: $g_0 = 2.0058$, $\Delta H_{pp} = 32.674$ Gs, $A_N = 15.81$ Gs (triplet peak in 1×10^{-4}M, CH_2Cl_2); MS (FAB) m/z (int.%): 725 (M^+, 48), 397 (100);

HRMS: m/z calcd. for $C_{38}H_{49}N_2O_{12}$: 727.3437 $[M + 2H]^+$, found: 727.3429 $[M + 2H]^+$.

Compound **10e:** Yield: 88%; mp 120–121°C; $[\alpha]_D^{20}$ −53 (c = 0.5, CH_2Cl_2); IR (KBr) (cm^{-1}): 3334 (NH), 1780 (lactone), 1719 (NHCO), 1485, 1506, 1589 (aromatic C=C), 929 (OCH_2O), 1125, 1177, 1239 (C–O), 1365 ($NO^{\cdot}$); ESR: $g_0 = 2.0058$, $\Delta H_{pp} = 27.404$ Gs, $A_N = 15.81$ Gs (triplet peak in 1×10^{-4} M, CH_2Cl_2); MS (FAB) m/z(int.%): 759 (M^+, 27), 397 (100); HRMS: m/z calcd. for $C_{41}H_{47}N_2O_{12}$: 761.3280 $[M + 2H]^+$; found: 761.3284 $[M + 2H]^+$.

General Procedure for Reduction of the Representative Compound 10e to Compound 11

To a solution of 5 mg of compound **10e** in 0.3 ml of $CDCl_3$ in an NMR tube was added 0.5 ml of 0.03 M phenylhydrazine in $CDCl_3$. After 10 min at 25°C under nitrogen, the progress of the reduction could be monitored visually by the disappearance of the red-orange color of compound **10e**. It gave the corresponding N-hydroxy amine **11**. Compound **11** was characterized by IR, ^{1}H NMR, and MS (FAB) as rapidly as possible.

IR (KBr) (cm^{-1}): 3336 (NH, OH, s), 1773 (lactone), 1708 (NHCO), 1485, 1594, 1600 (aromatic C=C), 933 (OCH_2O), 1126, 1179, 1241 (C–O); ^{1}H NMR ($CDCl_3$) δ_H: 1.18 (6H, s, $2CH_3$ in tetramethylpiperidine ring), 1.21 (6H, s, $2CH_3$ in tetramethylpiperidine ring), 1.50–2.00 (4H, m, $2CH_2$ in tetramethylpiperidine ring), 2.52 (1H, m, 3-H), 2.66 (1H, dd, $J = 14.0$ Hz, 4.4 Hz, 2-H), 3.05 (2H, m, Ph-$\underline{CH_2}$), 3.70 (6H, s, 3′,5′-OCH_3), 3.79 (3H, s, 4′-OCH_3), 3.94 (6H, d, $J = 8.8$ Hz, 11-H), 4.01 (1H, m, CH in tetramethylpiperidine ring), 4.49 (1H, d, $J = 4.4$ Hz, 1-H), 4.67 (1H, dd, $J = 10.2$ Hz, 6.6 Hz, 11″-H), 4.96 (1H, m, Ph-CH_2-$\underline{CH}$), 5.87 (1H, d, $J = 9.2$ Hz, 4-H), 5.98 (2H, d, $J = 6.0$ Hz, OCH_2O), 6.32 (2H, s, 2′,6′-H), 6.47(1H, s, 8-H), 6.67(1H, s, 5-H), 7.11(5H, s, $\underline{Ph}$-CH_2); MS(FAB) m/z (int.%): 761 ($[M + 1]^+$, 27), 397 (100).

Biological Assay

Compounds **10a**–**10e** were dissolved in Me_2SO at a concentration of 20 mM, as the stock solution was diluted before use with H_2O to the desired concentration of each compound. The cells were grown in RPMI 1640 medium with 10% fetal-calf serum, penicillinG (100 IU/ml) and streptomycin (100 μg/ml) at 37°C under a humidified 95% air +5% CO_2 atmosphere. Assays for the cytotoxicity in P-388 murine leukemia and A-549 human lung carcinoma cells were carried out according to the procedures described by Bergeron.The prototypical inhibitor etoposide was included as reference standard; the results of these assays were used to obtain the corresponding inhibition rates.

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