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Preliminary communication

First synthesis and biological evaluation of novel spin-labeled derivatives of deoxypodophyllotoxin

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ABSTRACT

Deoxypodophyllotoxin inhibits tubulin polymerization and induces cell cycle arrest at G2/M, followed by apoptosis. In order to find compounds with superior bioactivity and less toxicity, a series of spin-labeled derivatives of deoxypodophyllotoxin were synthesized by reacting 4′-demethyl-4-deoxypodophyllotoxin (DPPT) with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl) amino acids. The cytotoxic activities against three tumor cell lines (HL-60, RPMI-8226, A-549) in vitro and the antioxidative activities in tissues of Sprague Dawley (SD) rats of target compounds were evaluated, and the results indicated that compounds **11a**–**h** were more potent in terms of cytotoxicities and antioxidative activities than either parent compound DPPT or anticancer drug VP-16.

1. Introduction

Podophyllotoxin (PT, **1**) and its many related derivatives are well known to have pronounced antineoplastic and anti-viral properties [1]. PT preferentially binds to tubulin and inhibits tubulin polymerization, while some semi-synthetic derivatives of PT, such as etoposide (VP-16), teniposide (VM-26) and etopophos, have potent inhibitory activity of DNA topoisomerase II. And these derivatives of PT currently used in chemotherapy for various types of cancer, including small cell lung cancer, testicular carcinoma, and Kaposi's sarcoma [2–9]. Deoxypodophyllotoxin (DPT, **2**), as an analogue of PT, has antiproliferative and antitumor activities in diverse cell types [10–14], as well as anti-inflammatory [15] and anti-viral [16] activities. The mechanism studies showed that DPT inhibits tubulin polymerization and induces cell cycle arrest at G2/M, followed by apoptosis through multiple cellular processes, involving the activation of ATM, upregulation of p53 and Bax, activation of caspase-3 and -7, and accumulation of PTEN resulting in the inhibition of the Akt pathway [17,18]. Recently, Ahn and co-workers synthesized many derivatives of DPT, such as alkyl, carboxylalkyl esters of 4′-demethyl-4-deoxypodophyllotoxin (DDPT, **3**) and prodrugs of DPT including carbamates and carbonate. The antitumor activities of all these compounds showed that most derivatives enhanced cytotoxic or antitumor activity compared with that of parent compound DPT [19,20].

The nitroxyl free radicals have a wide range of activities in biology. Previous studies have shown that the introduction of nitroxyl moiety can lead to a series of positive effects, such as higher alkylating, lower carbamoylating activity, better antimelanomic activity and lower general toxicity. These types of compounds can move through cell membranes as a transport [21–23]. In addition, the nitroxyl free radicals possess low toxicity and are not mutagenic or carcinogenic by themselves [24]. Our group had been aiming at the discovery of new drugs of PT derivatives with improved bioactivities and less toxicity in recent years [25–34]. A series of spin-labeled podophyllotoxin analogues, *via* modification of different positions, had been prepared and these compounds showed significant antitumor activity with marked decrease in toxicity when compared with the parent compound [25–28]. Especially, GP-11 (**4**) (Fig. 1) was reported as low immunosuppressive antitumor agent, which increased the mitotic index and resulted in G2/M phase, and to a lesser extent, S arrest. This compound has the possibility of becoming a promising new antitumor drug. Inspired by previous work as well as the fact that L-amino acids, possessing good water solubility if actively transplanted into mammalian tissue, are often used as carrier vehicles

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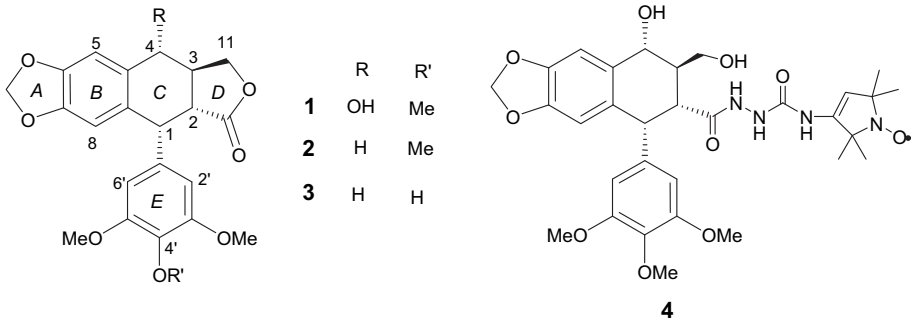


Fig. 1. Structures of podophyllotoxin (**1**) and related compounds.

for some drugs, a series of spin-labeled derivatives of DDPT were synthesized and the cytotoxic activities against three tumor cells (A-549, RPMI-8226 and HL-60) were evaluated. We also determined malondialdehyde (MDA) on liver and kidney homogenate of SD rats by the TBA method.

2. Chemistry

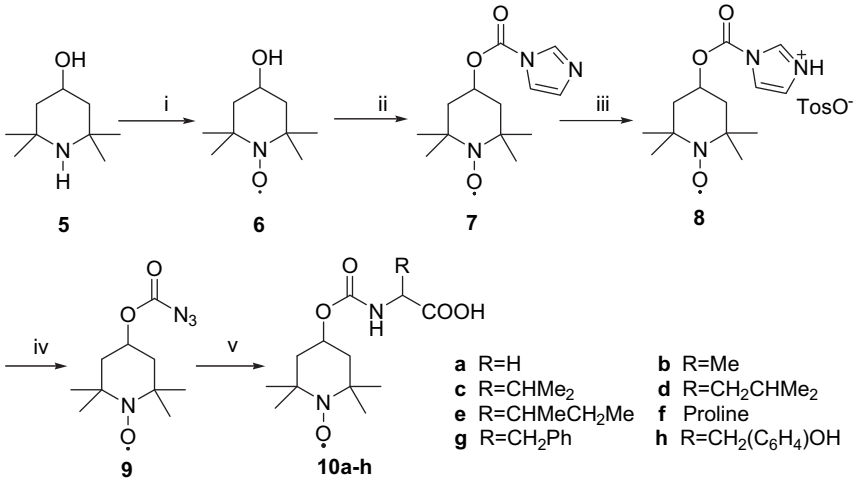
The synthesis of compounds **10a**–**h** is outlined in Scheme 1. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**6**) was prepared in excellent yield by means of oxidation of 4-hydroxyl-2,2,6,6-tetramethylpiperidine (**5**) with sodium tungstate–hydrogen peroxidate–EDTA [35]. Then the compound **6** reacted with *N*,*N*′-carbonyldiimidazole to give the intermediate *N*-(1-oxyl-2,2,6,6-tetramethyl- piperidinyloxycarbonyl)-imidazole (**7**) [36]. The intermediate **7** without isolation, further reacted with *p*-toluenesulfonic acid monohydrate to give its higher reactive tosylate (**8**). The salt **8** was so reactive that it was instantaneously converted into compound **9** when dissolved in an aqueous solution of sodium azide. Compounds **10a**–**h** were obtained in good yield by reaction of the alkoxycarbonyl azide (**9**) with free amino acids in the presence of MgO [37].

The synthetic route to the target compounds **11a**–**h** involved the intermediate 4′-demethyl-4-deoxypodophyllotoxin **3** which was prepared from **1**. Compound **1** was isolated from a Chinese medicinal herb *Podophyllum emodi* Wall var Chinesis Sprague (Scheme 2). The intermediate **3** was prepared from **1** through successive 4-deoxylation by hydrogenolysis [38] and 4′-demethylation with methanesulfonic acid/sodium iodide [39]. Then **3** was condensed with the appropriate **10a**–**h** in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and catalytic amount of 4-dimethylamino pyridine (DMAP) to provide the target compounds **11a**–**h**. The structures of the target compounds were identified by HRMS, ESR and IR spectral analysis.

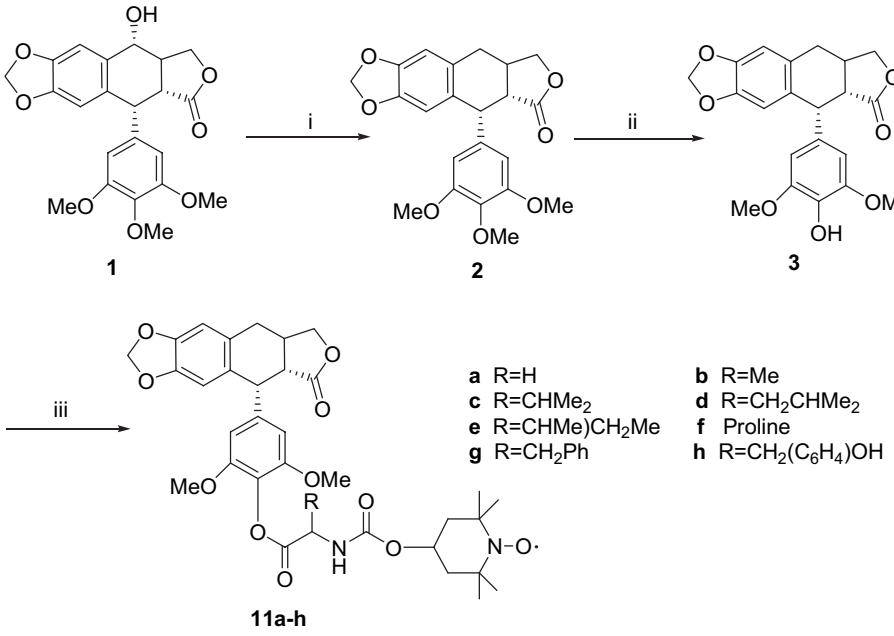
3. Biological results and discussion

The biological activities of this series of spin-labeled derivatives of DDPT **11a**–**h** were evaluated by an in vitro cytotoxicity test, which was carried out with a panel of three tumor cell lines that comprise human premyelocytic leukemia (HL-60), human multiple myeloma (RPMI-8226) and human lung carcinoma (A-549). The antioxidative activity on liver and kidney homogenate of SD rats was also tested and the results are summarized in Table 1.

As illustrated in Table 1, the spin-labeled derivatives of DDPT **11a**–**h** exhibited basically stronger inhibition of these three cell lines comparing to DDPT and VP-16. In these three tumor cell lines, the inhibition of all these spin-labeled compounds showed decreased activity in the following order: HL-60 > RPMI-8226 > A-549, such as, the IC_{50} value of compound **11b** is 0.012, 0.044 and 0.67 μM for HL-60, RPMI-8226 and A-549, respectively. In Table 1, we could also see that the target derivatives **11a**–**h** showed superior or comparable antioxidative activities in both tissues of kidney and liver homogenate of SD rats in comparison with parent compound DDPT and VP-16. The values of IC_{50} are ranged from 6.89 to 20.33 μM. However, different substituents at α-carbon of amino



Scheme 1. Reagents and conditions: (i) Na_2WO_4/H_2O_2/EDTA; (ii) *N*,*N*′-carbonyldiimidazole/THF; (iii) *p*-toluenesulfonic acid monohydrate; (iv) NaN_3/water, stir; (v) amino acids/MgO, stir, 24 h.



Scheme 2. Reagents and conditions: (i) Pd/C 10%, H_2; (ii) $MeSO_3H$/NaI, CH_2Cl_2; (iii) **10a–h**, DCC, DMAP, CH_2Cl_2, N_2.

Table 1
Biological activity of compounds **11a–h**.

Compounds	Cytotoxicity (IC_{50}, μM)[a]			Antioxidate activity (IC_{50}, μM)[a]	
	A-549[b]	RPMI-8226[c]	HL-60[c]	Kidney	Liver
11a	0.62	0.073	0.0087	13.70	14.37
11b	0.67	0.044	0.012	15.78	15.32
11c	0.55	0.16	0.024	20.33	19.30
11d	0.77	0.11	0.085	12.08	18.01
11e	0.83	0.058	0.036	6.89	13.16
11f	0.60	0.13	0.14	14.56	9.70
11g	0.50	0.031	0.018	15.05	10.38
11h	0.27	0.043	0.032	16.27	15.2
DDPT	0.80	0.23	0.11	22.99	33.75
VP-16	0.83	0.70	0.18	64.63	46.38

[a] The value is the average of triplicate.
[b] MTT method, drugs exposure was for 48 h.
[c] CCK-8 method, drugs exposure was for 72 h.

acid in this class of compounds did not show obviously different effects on the inhibition of the three tumor cell lines in vitro and on antioxidative activity.

These results indicated 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 and antioxidant activities. As can be seen, as a whole, the introduction of a stable nitroxyl radical into the molecule of **3** with L-amino acids led to potentiate their antitumor and antioxidative activity, which also indicated that the design and synthesis of these compounds should be beneficial for therapeutic values of deoxypodophyllotoxin and they probably have synergistic action to tumor cell lines.

4. Conclusion

In summary, novel spin-labeling of podophyllotoxin class is a promising direction in antitumor chemotherapy, not only because these compounds exhibit superior activities, but also because they can be monitored by ESR in pharmacological experiments. In this reported work, eight spin-labeled derivatives of deoxypodophyllotoxin were first synthesized successfully, the results of biological evaluation showed that the vast majority of compounds exhibited more potent cytotoxicities against HL-60, RPMI-8226 and A-549. Compound **11a** showed most potent cytotoxicity against HL-60 with IC_{50} at 0.0087 μM. In addition, the synthesized derivatives **11a–h** showed either similar or better antioxidative activities in both tissues of kidney and liver homogenate of SD rats than that of the parent compound DDPT, and anticancer drug VP-16. Further biological evaluation is in progress to define their tubulin polymerization activity and to clarify whether spin-labeled deoxypodophyllotoxin analogues might counteract the toxicity of this class antitumor drug.

5. Experimental

Melting points were determined in Kofler apparatus and were uncorrected. IR spectra were measured on a Nicolet 5DX-FT-IR spectrometer on neat samples placed between KBr plates. ^1H NMR spectra were obtained by using a Bruker AM400 with TMS as an internal standard, all chemical shifts are reported as δ ppm. Mass spectra were recorded on a Bruker Daltonics APEXII49e spectrometer with ESI source as ionization. Optical rotations were measured on a Perkin Elmer 341 polarimeter in a 1 dm cell at 23 °C. The electron spin resonance (ESR) spectra were obtained with a Bruker A300 X-band EPR spectrometer. The *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-amino acids **10a–h** used for the experiments were prepared by following a modified previous procedure [27,35–37].

5.1. Synthesis of DDPT (*3*)

A mixture of 10% Pd/C (6.0 g) in acetic acid (150 mL) was stirred under hydrogen until no further hydrogen was absorbed. Then PT (8.0 g) was added and the reaction mixture was stirred for another 5 h at 95 °C under 2 atm of hydrogen. The mixture was filtered and the filtrate was evaporated. The residue was purified by column chromatography on silica gel (elution with CH_2Cl_2) yielding DPT (5.6 g, yield 73%).

To a solution of DPT (3.98 g, 10 mmol) in dry CH_2Cl_2 (100 mL), NaI (4.47 g, 30 mmol) was added and stirred for 5 min. To this

stirred suspension, $MeSO_3H$ (2.88 g, 30 mmol) was added dropwise with syringe at 0 °C and stirring was continued for another 5 h at room temperature. Nitrogen was bubbled through the solution to drive off the excess hydrogen iodide. The solution was then evaporated in vacuo and purified by chromatography to give yellow oil. Further purification by recrystallization with methanol afforded 2.1 g of **3** as a white solid. Yield 55%; ^1H NMR (400 MHz, $CDCl_3$): δ 6.66 (s, 1H), 6.52 (s, 1H), 6.35 (s, 2H), 5.94 (d, $J = 9.2$ Hz, 2H), 5.41 (s, 1H), 4.60 (s, 1H), 4.45–4.42 (m, 1H), 3.93–3.90 (m, 1H), 3.78 (s, 6H), 3.08–3.05 (m, 1H), 2.77–2.72 (m, 3H).

5.2. General procedure of synthesis of **11a–h**

A mixture of the appropriate **10a–h** (0.5 mmol), DDPT (0.192 g, 0.5 mmol) and DMAP (20 mg) was stirred in dry CH_2Cl_2 (10 mL) for 5 min at room temperature under nitrogen. *N*,*N*-Dicyclohexylcarbodiimide (DCC, 106 mg, 0.5 mmol) was added and the mixture was stirred for 4 h. The reaction mixture was filtered and the filtrate was evaporated. The residue was separated by column chromatography on silica gel with CH_2Cl_2–acetone to afford compounds **11a–h**.

5.2.1. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-glycin 4′-demethyl-4-deoxypodophyllic ester (**11a**)*

The yield: 65%; red powder; mp: 149–152 °C; [α] −65 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3329, 2933, 2849, 1778, 1723, 1507, 1484, 1462, 1337 (N–O.), 1227, 1130, 931; MS *m*/*z* 639 ($M + 2H^+$); ESR: An = 16.02 G, $g_0 = 2.0061$; HRMS (ESI) 662.2446 for $[M + Na]^+$ (calcd 662.2436 for $C_{33}H_{39}O_{11}N_2$).

5.2.2. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-alanine 4′-demethyl-4-deoxypodophyllic ester (**11b**)*

The yield: 69%; red powder; mp: 139–142 °C; [α] −67 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3328, 2932, 2849, 1773, 1720, 1507, 1484, 1461, 1338 (N–O.), 1227, 1130, 930; MS *m*/*z* 653 (M^+); ESR: An = 16.16 G, $g_0 = 2.006$; HRMS (ESI) 676.2603 for $[M + Na]^+$ (calcd 676.2614 for $C_{34}H_{41}O_{11}N_2$).

5.2.3. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-valine 4′-demethyl-4-deoxypodophyllic ester (**11c**)*

The yield: 75%; red powder; mp: 138–140 °C; [α] −67 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3329, 2932, 2850, 1771, 1721, 1507, 1484, 1463, 1338 (N–O.), 1227, 1130, 936; MS *m*/*z* 682 ($M + H^+$); ESR: An = 15.98 G, $g_0 = 2.006$; HRMS (ESI) 704.2916 for $[M + Na]^+$ (calcd 704.2924 for $C_{36}H_{45}O_{11}N_2$).

5.2.4. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-leucine 4′-demethyl-4-deoxypodophyllic ester (**11d**)*

The yield: 63%; red powder; mp: 139–143 °C; [α] −68 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3335, 2934, 2873, 1773, 1722, 1507, 1484, 1463, 1338 (N–O), 1226, 1130, 935; MS *m*/*z* 695 ($M + 2H^+$); ESR: An = 16.04 G, $g_0 = 2.0061$; HRMS (ESI) 718.3072 for $[M + Na]^+$ (calcd 718.3066 for $C_{37}H_{47}O_{11}N_2$).

5.2.5. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-isoleucine 4′-demethyl-4-deoxypodophyllic ester (**11e**)*

The yield: 42%; red powder; mp: 138–140 °C; [α] −59 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3335, 2934, 1771, 1721, 1507, 1484, 1462, 1338 (N–O.), 1227, 1130, 936; MS *m*/*z* 695 (M^+); ESR: An = 16.10 G, $g_0 = 2.006$; HRMS (ESI) 718.3072 for $[M + Na]^+$ (calcd 718.3075 for $C_{37}H_{47}O_{11}N_2$).

5.2.6. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-proline 4′-demethyl-4-deoxypodophyllic ester (**11f**)*

The yield: 45%; red powder; mp: 148–152 °C; [α] −84 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3327, 2931, 2850, 1774, 1706, 1506, 1484, 1462, 1342 (N–O.), 1226, 1129, 926; MS *m*/*z* 679 (M^+); ESR: An = 15.95 G, $g_0 = 2.006$; HRMS (ESI) 702.2759 for $[M + Na]^+$ (calcd 702.2763 for $C_{36}H_{43}O_{11}N_2$).

5.2.7. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-phenylalanine 4′-demethyl-4-deoxypodophyllic ester (**11g**)*

The yield: 54%; red powder; mp: 119–126 °C; [α] −62 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3330, 2933, 2848, 1773, 1721, 1506, 1484, 1460, 1338 (N–O.), 1226, 1129, 936; MS *m*/*z* 731 ($M + 2H^+$); ESR: An = 15.78 G, $g_0 = 2.0061$; HRMS (ESI) 752.2914 for $[M + Na]^+$ (calcd 752.2916 for $C_{40}H_{45}O_{11}N_2$).

5.2.8. *N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-L-tyrosine 4′-demethyl-4-deoxypodophyllic ester (**11h**)*

The yield: 44%; red powder; mp: 166–168 °C; [α] −80 (*c* 0.1, $CHCl_3$); IR (cm^{-1}) 3433, 2974, 2937, 1766, 1723, 1508, 1485, 1463, 1341 (N–O.), 1234, 1130, 937; MS *m*/*z* 747 ($M + 2H^+$); ESR: An = 15.88 G, $g_0 = 2.006$; HRMS (ESI) 745.2977 for $[M + Na]^+$ (calcd 745.2967 for $C_{40}H_{45}O_{11}N_2$).

5.3. Cytotoxicity assays

Cells were incubated at 37 °C in a 5% CO_2 atmosphere. The MTT and Cell Counting Kit-8 (CCK-8) colorimetric assay were used to determined growth inhibition [40]. The spin-labeled derivatives were dissolved in saline for five concentrations (0.005–50 μM). For the HL-60 and RPMI-8226, cells were plated in 96-well plates and exposed in quadplex well for 48 or 72 h. Then the CCK-8 was added to each well. After 4 h of incubation, the absorbance at λ_{450} was determined with a plate reader. For the A-549, cells were plated in 96-well plates and allowed to attach for 24 h, then exposed in quadplex well for 48 h. The media were aspirated, and 10 μL of 5 mg/mL MTT solution (dilute in sterile PBS) diluted in serum-free media was added to each well. After 4 h of incubation, the solution was centrifuged for 10 min under 2000 rpm. The supernatant was mixed with 150 μL DMSO, then was shaken on an oscillator. The absorbance at λ_{570} was determined on a plate reader. IC_{50} values were determined from a log plot of percent of control versus concentration.

5.4. In tissues antioxidative activity

The extent of lipid peroxidation was assessed using a modification of the thiobarbituric acid (TBA) assay [41]. Determination of malondialdehyde (MDA) content was carried out in 5% and 1% of liver and kidney homogenate of SD rats in 0.9% NaCl solution, respectively. The tissue homogenate 1 mL was incubated with test compounds or vehicle control (DMSO 2.5 mL) at 37 °C for 10 min incubation, MDA was determined by the TBA colormetric analysis method. The absorbance at λ_{532} was determined on a UV spectrophotometer. IC_{50} values were determined from 50% of control versus concentration.

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