Full Paper

Synthesis and Biological Evaluation of 4α/4β-Imidazolyl Podophyllotoxin Analogues as Antitumor Agents

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A series of 4α/4β-imidazolyl podophyllotoxin analogues have been designed and synthesized. All of the compounds were evaluated for their anticancer activity against a panel of three human cancer cell lines. Within the cell lines tested, some of the synthesized compounds showed promising anticancer activity. Compound **12**, in particular, exhibited remarkable cytotoxicity, demonstrating effects against all tumor cell lines, including the K562/ADM cell line.

Keywords: Cancer cell lines / Cytotoxicity / Multidrug resistance / Podophyllotoxin

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Introduction

Podophyllotoxin (**1**) has been shown to inhibit tubulin assembly during the formation of microtubules *via* tubulin binding [1, 2]; however, its high toxicity has limited its application as a chemotherapeutic. In order to obtain more potent, yet less toxic, anticancer agents, many derivatives of podophyllotoxin have been synthesized. Among them, two semisynthetic glycosidic cyclic acetals of podophyllotoxin, etoposide (**2**) and teniposide (**3**), are widely used as anticancer drugs and show encouraging clinical efficacy against several types of neoplasms [3, 4]. This clinical efficacy is due to their ability to inhibit the ubiquitous DNA-topoisomerase II [5, 6]. However, both drugs have some intrinsic disadvantages such as developed drug resistance, myelosuppression and poor oral bioavailability. Another podophyllum glycoside derivative, NK611 (**4**), is currently being evaluated in phase I clinical trials [7]. The main advantage of this compound is its superior water solubility compared to that of etoposide.

For the last two decades, synthetic studies on podophyllotoxin have been focused on C-4 non-sugar substituted analogues, which have proven to be capable of overcoming drug resistance against etoposide. GL-331 (**5**) [8–10], a 4β-arylamino derivative, was more active than etoposide in both *in-vitro* and *in-vivo* studies, especially in resistant malignancies. TOP-53 (**6**) [11–13], a 4β-alkylated etoposide analogue, exhibited high activity against non-small cell lung cancer in both tumor cell lines and animal tumor models. Recently, more complex and diverse modifications to podophyllotoxin have been achieved. Among them, Wang [14, 15] and Kumar [16–18] have reported the synthesis of 4β-[(5-substituted)-1,2,3-triazol-1-yl] and 4β-[(4-substituted)-1,2,3-triazol-1-yl] podophyllotoxin derivatives as new anticancer compounds, respectively (Fig. 1). These two series of podophyllotoxin derivatives showed significant cytotoxic activity against several human cancer cell lines. These results attracted our interest and prompted us to synthesize the C-4 imidazole substituted analogues as new anticancer compounds. Thus, we now report the synthesis of a library of 4α/4β-imidazolyl podophyllotoxin derivatives as antitumor agents.

Results and discussion

Chemistry

The synthetic route to the target compound formation is depicted in Scheme 1. Compounds **10–13** were obtained

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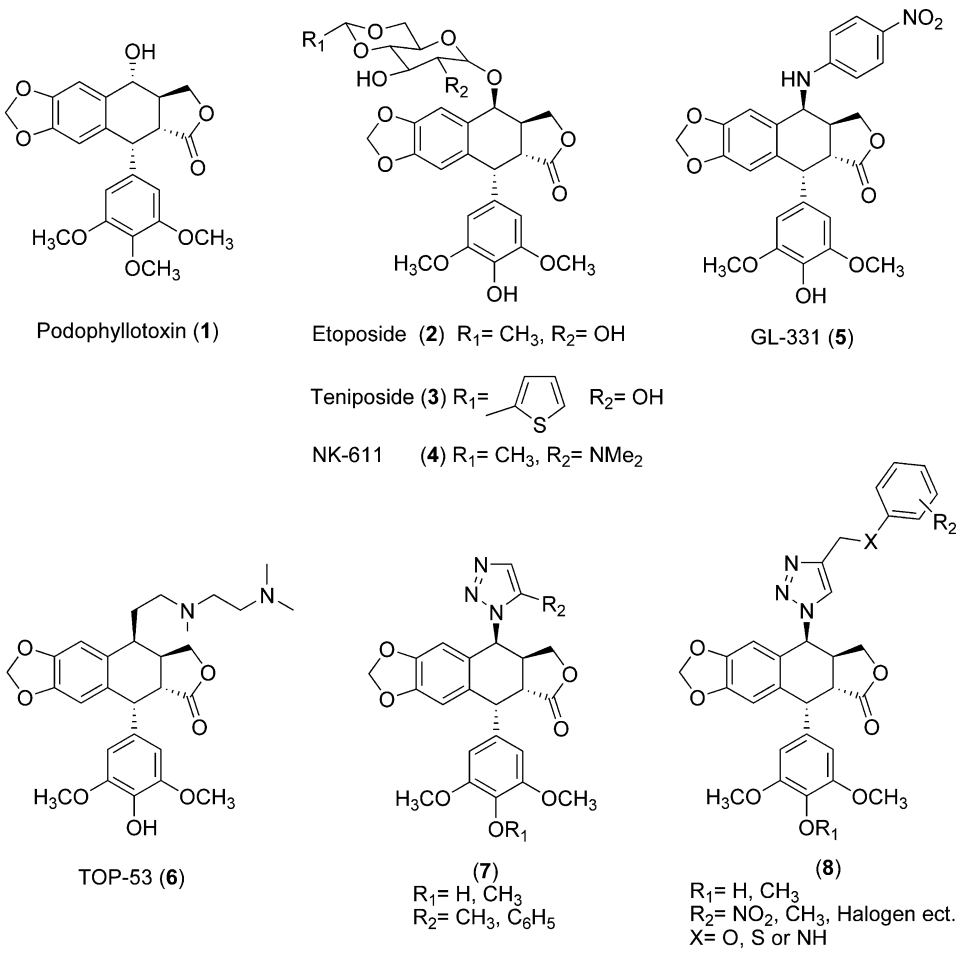
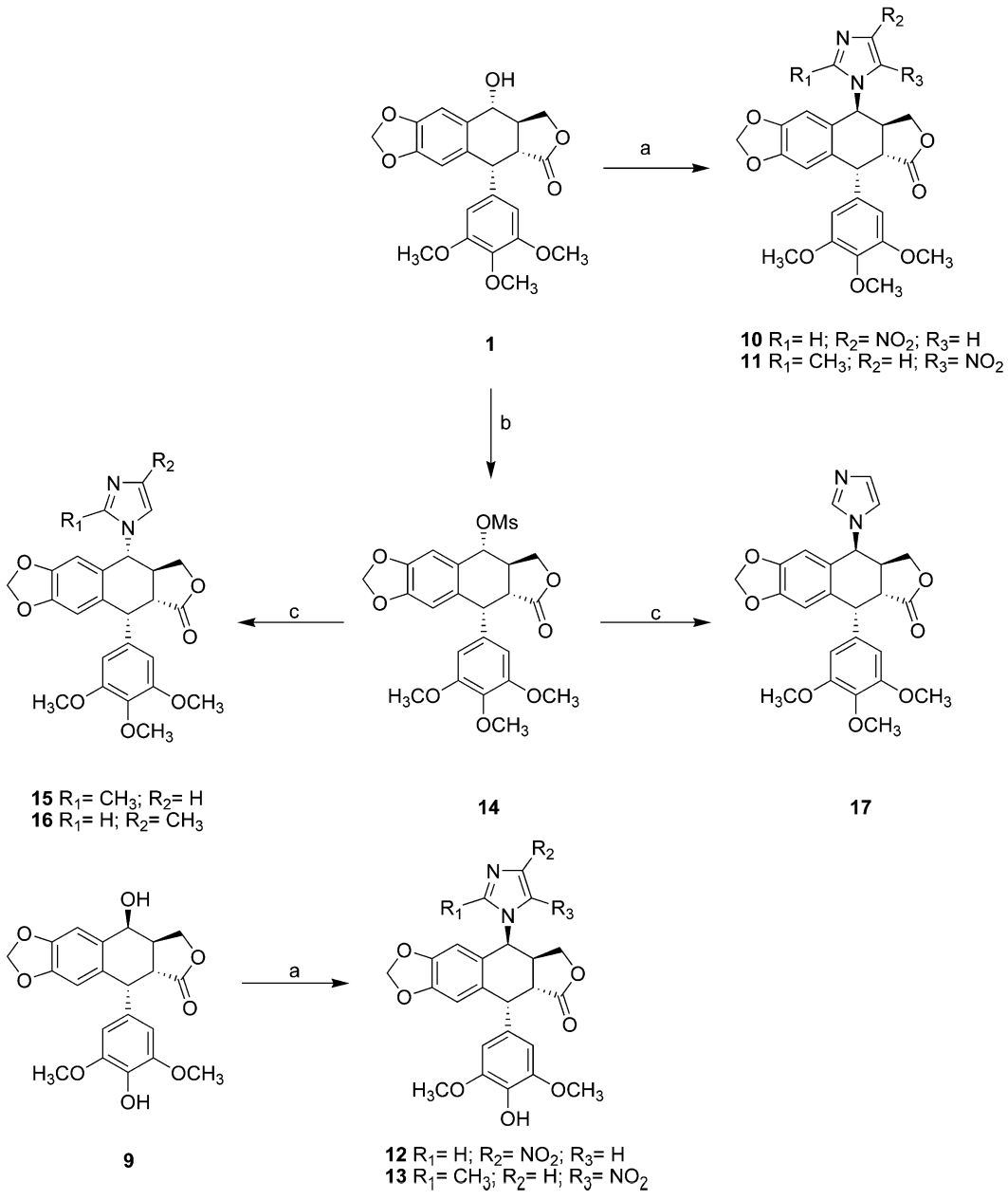


Figure 1. Structures of podophyllotoxin, etoposide and other analogues.

through the coupling of podophyllotoxin (**1**) or 4'-*O*-demethylepipodophyllotoxin (**9**) with substituted imidazoles and by employing anhydrous $FeCl_3$ in CH_3NO_2 under 50–60°C with good yields [19]. However, the above reaction condition was only suitable for imidazoles with electron-withdrawing groups. The attempt to obtain the derivatives of imidazoles with electron-donating groups was unsuccessful. Therefore, compounds **15–17** were prepared from compound **1** through mesylation [20] with mesyl chloride in the presence of triethylamine, followed by nucleophilic substitution with imidazole or imidazoles with electron donor groups. The reaction of mesylate **14** with the corresponding substituted imidazoles yielded two major products. We speculated that they were a pair of configurational isomers (C4-α and C4-β imidazolyl podophyllotoxin derivatives), presumably formed via an SN_1 mechanism. Purification of these configurational isomers was extremely difficult owing to their similar polarities. Compounds **15–17** were obtained by recycling preparative HPLC, but their C-4 isomers were not. The structures of all target compounds were identified by ^{1}H-NMR, ^{13}C-NMR, IR and HRMS.

During synthesis of the target compounds **10–13**, the C-4β isomers were obtained as the major products, and in some cases, C-4α isomers were also observed in trace amounts. The reaction was found to be highly stereoselective. We presumed that one of the reasons for this stereoselectivity was that the bulky C-4α pendant aromatic ring E directed the substituent to bind in the opposite plane and yield a C-4β isomer as the major product. Another possible reason was that imidazole could coordinate to $FeCl_3$. The subsequent bulky complex led to a greater spatial hindrance and thus led to greater stereoselectivity. The complex might also affect reactivity, which is why this reaction condition was only suitable for imidazoles with electron-withdrawing groups but not for imidazoles with electron-donating groups. Further study of this reaction is currently underway in our group, and the corresponding research will be reported separately in due course.



Scheme 1. Synthesis of compounds **10–13** and **15–17**. Reaction conditions: (a) Anhydrous $FeCl_3$, CH_3NO_2, and 4-nitro-1*H*-imidazole or 2-methyl-5-nitro-1*H*-imidazole; (b) MsCl, Et_3N, CH_2Cl_2; (c) CH_3CN, 1*H*-imidazole or 2-methyl-1*H*-imidazole or 4-methyl-1*H*-imidazole.

The assignment of the configuration at the C-4 position for target podophyllotoxin derivatives was based on $J_{3,4}$ coupling constants or NOESY. Compound **15** has a value of 11.0 Hz for $J_{3,4}$, and the NOESY spectra reveal that an obvious coupling between H-4 and H-1 is observed due to a *trans*-relationship between H-3 and H-4. On the other hand, compound **10** has a value of 5.0 Hz for $J_{3,4}$, and there is a correlation between H-4 and H-2′, 6′ as H-3 is *cis* to H-4. The C-4 configuration of the other compounds was confirmed by the same method.

Cytotoxic activity

The biological activities of 4α/4β-imidazolyl podophyllotoxin derivatives were evaluated by their cytotoxic activitiy against three tumour cell lines, which comprised the following:

Table 1. *In vitro* cytotoxicity of compounds **10–13** and **15–17** against a panel of human tumor cell lines (IC_{50}, μM).

Compound	HeLa	K562	K562/ADM
10	>10	>10	>10
11	>10	>10	>10
12	8.12 ± 1.03	7.43 ± 0.62	4.16 ± 0.54
13	>10	>10	>10
15	7.46 ± 2.19	8.85 ± 2.56	>10
16	4.48 ± 1.67	8.08 ± 0.90	>10
17	5.22 ± 0.96	2.88 ± 1.36	>10
2	2.99 ± 0.87	6.0 ± 1.84	76.55 ± 8.36

HeLa (cervix, human), K562 (leukemia, human) and K562/ADM (adriamycin resistant), with etoposide serving as the reference compound. The screening procedure was based on the standard MTT method. The IC_{50} values are shown in Table 1.

Some of the compounds exhibited promising antitumor potency against one or more cell lines. For the HeLa cell line, compounds **12**, **15**, **16** and **17** demonstrated cytotoxicity at a concentration below 10 μM. Compounds **10**, **11** and **13** were less potent, requiring concentrations above 10 μM to initiate cytotoxicity. For the K562 cell line, compound **17** was found to be more potent than etoposide, while compounds **12**, **15** and **16** were as effective as etoposide. The other three compounds showed limited effects. For the K562/ADM cell line, compound **12** displayed remarkable anticancer activity, which was at least one order of magnitude more potent than etoposide. However, the other compounds were less cytotoxic, requiring concentrations above 10 μM.

For compounds **15** and **16**, the only difference was the position of methyl in the imidazole group. It was demonstrated that pharmacologically, the different positions of methyl in the imidazole group did not affect the overall cytotoxicity. Compounds **10** and **12** shared very similar structures, with the exception of the 4′-methoxy moiety. There was a distinction between the activity of compounds **10** and **12**, indicating that demethylation of the 4′-methoxy moiety markedly affected the overall activity profile. For the 4′-*O*-methyl-4β-imidazolyl analogues of podophyllotoxin (compounds **10** and **11**), pharmacological results showed that nitro and methyl substituents on the imidazole ring reduced the overall activity.

In conclusion, seven podophyllotoxin derivatives were synthesized and screened for anticancer activities against three human cancer cell lines. Some compounds exhibited *in-vitro* cytotoxic activitiy, particularly compound **12**. Thus, the results obtained with the newly synthesized podophyllotoxin derivatives demonstrate that some derivatives warrant the pursuit of additional research. Additional studies are currently being conducted to determine the actions of these compounds on tubulin and human DNA topoisomerase II. Other syntheses and *in-vivo* antitumor activity studies are also currently in progress.

Experimental

Chemistry

All commercially available reagents and solvents were employed without further purification. Melting points were determined in a Büchi Melting Point B-540 apparatus and were uncorrected. Infrared spectra were recorded in a Bruker IFS-55 spectrophotometer. Optical rotations were measured on a Perkin Elmer 241MC spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in Bruker ARX 300 MHz or 600 MHz, using TMS as the internal standard. Chemical shift values were reported in δ (ppm) and coupling constants in Hz. High resolution mass spectrometry (HRMS) was obtained using a Bruker MicroTOF QII Time of Flight mass spectrometer. Recycling preparative HPLC was obtained from a reverse phase column (JAIGEL ODS-AP-L SP-120-15), using JAI LC-9103. Column chromatography was performed on silica gel H and analytical TLC data on silica gel HF_{254}.

*General procedure for preparation of compounds **10–13***

To a stirred solution of podophyllotoxin **1** (1 mmol) or 4′-*O*-demethylepipodophyllotoxin **9** (1 mmol) and corresponding imidazole (1 mmol) in dry nitromethane (3 mL) was added anhydrous $FeCl_3$ (0.1 mmol). The reaction mixture was stirred under 60°C until the starting material had been completely converted. The reaction mixture was then concentrated under reduced pressure and the residue was purified by column chromatography on silica gel with CH_2Cl_2/AcOEt to give compounds **10**–**13**.

*4β-(4-Nitro-1H-imidazol-1-yl)-4-desoxypodophyllotoxin **10***

Pale yellow solid; yield: 79.4%; mp: 235.4–237.0°C; $[\alpha]_D^{25}$ −70.4 (*c* 0.5, CH_2Cl_2); ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 2.94 (dd, 1H, J = 5.0, 14.5 Hz), 3.19–3.25 (m, 1H), 3.39 (dd, 1H, J = 9.0, 10.8 Hz), 3.77 (s, 6H), 3.82 (s, 3H), 4.42 (t, 1H, J = 8.0 Hz), 4.78 (d, 1H, J = 5.0 Hz), 5.70 (d, 1H, J = 5.0 Hz), 6.04 (s, 2H), 6.31 (s, 2H), 6.62 (s, 1H), 6.66 (s, 1H), 7.36 (s, 1H), 7.55 (s, 1H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 36.9, 41.2, 43.5, 56.4, 57.0, 60.8, 66.8, 102.2, 108.3, 108.6, 110.5, 119.0, 123.7, 133.4, 133.8, 135.8, 137.8, 148.3, 148.6, 149.9, 152.9, 172.2; IR (KBr): 3435.8, 2904.5, 1783.6, 1589.4, 1507.6, 1486.0, 1237.8, 1125.5, 1093.2, 1036.8, 1002.3 cm^{-1}; ESI-HRMS: Calcd. for $C_{25}H_{23}N_3O_9Na$, 532.1327; found, 532.1326 $[M+Na]^+$.

*4β-(2-Methyl-5-nitro-1H-imidazol-1-yl)-4-desoxypodophyllotoxin **11***

Pale yellow solid; yield: 69.2%; mp: 272.2–273.6°C; $[\alpha]_D^{25}$ −59.8 (*c* 0.5, CH_2Cl_2); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.53 (s, 3H), 3.00 (dd, 1H, J = 4.9, 14.0 Hz), 3.16–3.36 (m, 2H), 3.77 (s, 6H), 3.82 (s, 3H), 4.42 (t, 1H, J = 7.3 Hz), 4.78 (d, 1H, J = 4.9 Hz), 5.55 (d, 1H, J = 4.8 Hz), 6.05 (s, 2H), 6.31 (s, 2H), 6.52 (s, 1H), 6.67 (s, 1H), 7.22 (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 13.9, 37.4, 41.3, 43.7, 55.5, 56.7, 61.1, 66.9, 102.5, 108.6, 108.8, 110.8, 119.1, 124.7, 133.5, 134.0, 138.2, 144.9, 147.2, 148.9, 150.1, 153.2, 172.4; IR (KBr): 3441.3, 2901.5, 1788.4, 1590.1,

1505.9, 1485.0, 1235.8, 1124.9, 1095.2, 1038.5, 1000.7 cm^{-1}; ESI-HRMS: Calcd. for $C_{26}H_{25}N_3O_9Na$, 546.1483; found, 546.1483 $[M+Na]^+$.

4′-O-Demethyl-4β-(4-nitro-1H-imidazol-1-yl)-4-desoxypodophyllotoxin **12**

White solid; yield: 76.0%; mp: 233.1–235.0°C; $[\alpha]_D^{25}$ −81.2 (*c* 0.5, CH_2Cl_2); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.06–3.19 (m, 1H), 3.21–3.26 (m, 1H), 3.33–3.36 (m, 1H), 3.65 (s, 6H), 4.41 (t, 1H, J = 7.5 Hz), 4.65 (d, 1H, J = 4.8 Hz), 5.94 (d, 1H, J = 4.3 Hz), 6.04 (s, 2H), 6.25 (s, 2H), 6.67 (s, 1H), 6.87 (s, 1H), 7.76 (s, 1H), 8.19 (s, 1H), 8.32 (s, 1H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 36.9, 40.8, 43.3, 56.2, 56.5, 67.7, 102.1, 108.9, 109.2, 110.3, 122.1, 125.9, 130.1, 134.4, 135.4, 137.9, 147.5, 147.6, 147.7, 148.8, 174.0; IR (KBr): 3422.3, 2902.3, 1770.6, 1612.6, 1506.7, 1483.9, 1230.7, 1113.8, 1095.5, 1031.7, 1003.2 cm^{-1}; ESI-HRMS: Calcd. for $C_{24}H_{21}N_3O_9Na$, 518.1170; found, 518.1169 $[M+Na]^+$.

4′-O-Demethyl-4β-(2-methyl-5-nitro-1H-imidazol-1-yl)-4-desoxypodophyllotoxin **13**

Pale pink solid; yield: 57.8%; mp: 290.4–291.5°C; $[\alpha]_D^{25}$ −54.2 (*c* 0.5, CH_2Cl_2); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.53 (s, 3H), 2.98 (dd, 1H, J = 5.2, 14.0 Hz), 3.17–3.36 (m, 2H), 3.81 (s, 6H), 4.40 (t, 1H, J = 7.6 Hz), 4.78 (d, 1H, J = 4.8 Hz), 5.47 (s, 1H), 5.54 (d, 1H, J = 4.6 Hz), 6.05 (s, 2H), 6.32 (s, 2H), 6.51 (s, 1H), 6.67 (s, 1H), 7.21 (s, 1H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 13.7, 37.0, 43.3, 54.6, 55.4, 56.5, 67.5, 102.1, 109.0, 109.5, 110.2, 121.6, 126.4, 130.1, 134.4, 135.4, 146.1, 146.3, 147.5, 147.7, 148.7, 174.0; IR (KBr): 3538.1, 3153.3, 2902.2, 1789.2, 1618.0, 1503.2, 1485.2, 1228.1, 1109.0, 1095.1, 1034.0, 1000.0 cm^{-1}; ESI-HRMS: Calcd. for $C_{25}H_{23}N_3O_9Na$, 532.1327; found, 532.1327 $[M+Na]^+$.

General procedure for preparation of compounds **15–17**

To a solution of podophyllotoxin **1** (1 mmol) in CH_2Cl_2 (10 mL) was added triethylamine (1.2 mmol) and mesyl chloride (1.2 mmol) at 0°C. Then the mixture was stirred for 0.5 h at room temperature. After washing with water and brine, the organic layer was dried over Na_2SO_4, concentrated under reduced pressure, and used for the next reaction without further purification. To the above crude intermediate **14** dissolved in dry CH_3CN (8 mL) was added corresponding imidazole (2 mmol) and the mixture was refluxed. While TLC analysis showed that the starting material had been completely converted, the reaction was stopped. The reaction mixture was diluted with CH_2Cl_2 and washed with water. The organic layers were dried over Na_2SO_4, concentrated under reduced pressure, and the residue purified by column chromatography on silica gel with AcOEt and further purified by recycling preparative HPLC to give compounds **15–17**.

4α-(2-Methyl-1H-imidazol-1-yl)-4-desoxypodophyllotoxin **15**

White solid; yield: 37.5%; mp: 242.7–243.7°C; $[\alpha]_D^{25}$ 5.4 (*c* 0.5, CH_2Cl_2); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.47 (s, 3H), 3.03–3.12 (m, 1H), 3.28 (dd, 1H, J = 5.2, 8.5 Hz), 3.86 (s, 6H), 3.88 (s, 3H), 4.08 (d, 1H, J = 9.9 Hz), 4.22 (d, 1H, J = 5.1 Hz), 4.30 (dd, 1H, J = 5.2, 10.0 Hz), 4.92 (d, 1H, J = 11.0 Hz), 5.88 (s, 2H), 5.91 (s, 1H), 6.38 (s, 1H), 6.48 (s, 2H), 6.85 (s, 1H), 7.14 (s, 1H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 13.6, 40.8, 43.5, 46.5, 55.1, 56.3, 60.9, 68.7, 101.4, 104.6, 105.7, 109.3, 116.2, 128.5, 129.2, 130.8, 137.3, 139.3, 146.1, 147.4, 147.7, 153.8, 176.6; IR (KBr): 3426.0, 2935.8, 1778.0, 1591.3, 1504.6, 1482.5, 1239.1, 1126.9, 1037.4, 1004.1 cm^{-1}; ESI-HRMS: Calcd. for $C_{26}H_{27}N_2O_7$, 479.1813; found, 479.1813 $[M+H]^+$.

4α-(4-Methyl-1H-imidazol-1-yl)-4-desoxypodophyllotoxin **16**

White solid; yield: 29.2%; mp: 225.8–227.3°C; $[\alpha]_D^{25}$ −63.6 (*c* 0.5, CH_2Cl_2); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.23 (s, 3H), 3.14–3.22 (m, 1H), 3.29 (dd, 1H, J = 4.9, 8.3 Hz), 3.85 (s, 6H), 3.87 (s, 3H), 4.13 (d, 1H, J = 10.0 Hz), 4.24 (d, 1H, J = 4.7 Hz), 4.32 (dd, 1H, J = 5.0, 10.0 Hz), 4.87 (d, 1H, J = 11.2 Hz), 5.84 (s, 1H), 5.90 (s, 2H), 6.38 (s, 1H), 6.48 (s, 2H), 6.99 (s, 1H), 7.60 (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 10.0, 40.4, 43.4, 46.6, 54.3, 56.2, 60.9, 68.7, 101.4, 104.5, 105.7, 109.3, 127.9, 128.1, 128.6, 130.6, 135.7, 137.3, 139.5, 147.5, 147.8, 153.8, 176.5; IR (KBr): 3422.4, 2938.5, 1779.3, 1588.0, 1505.2, 1484.0, 1235.0, 1127.0, 1092.7, 1035.9, 1002.4 cm^{-1}; ESI-HRMS: Calcd. for $C_{26}H_{27}N_2O_7$, 479.1812; found, 479.1814 $[M+H]^+$.

4β-(1H-Imidazol-1-yl)-4-desoxypodophyllotoxin **17**

White solid; yield: 43.0%; mp: 244.1–245.4°C; $[\alpha]_D^{25}$ −70.4 (*c* 0.5, CH_2Cl_2); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.02 (dd, 1H, J = 4.7, 14.2 Hz), 3.06–3.19 (m, 1H), 3.31 (t, 1H, J = 9.6 Hz), 3.76 (s, 6H), 3.82 (s, 3H), 4.35 (t, 1H, J = 7.0 Hz), 4.75 (d, 1H, J = 4.7 Hz), 5.60 (d, 1H, J = 4.8 Hz), 6.01 (s, 2H), 6.32 (s, 2H), 6.63 (s, 2H), 6.76 (s, 1H), 7.14 (s, 1H), 7.45 (s, 1H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 37.5, 41.8, 43.6, 55.4, 56.3, 60.7, 67.3, 101.9, 108.2, 109.0, 110.1, 119.1, 125.7, 130.2, 132.8, 134.3, 137.0, 137.6, 148.1, 149.1, 152.8, 173.1; IR (KBr): 3457.5, 2909.1, 1778.9, 1588.2, 1505.6, 1484.9, 1237.7, 1126.1, 1036.1, 1005.4 cm^{-1}; ESI-HRMS: Calcd. for $C_{25}H_{25}N_2O_7$, 465.1656; found, 465.1651 $[M+H]^+$.

In-vitro evaluation of cytotoxic activity

The antiproliferative activity of these podophyllotoxin derivatives against HeLa, K562 and K562/ADM cell lines was measured by the standard MTT assay. Cells were seeded in 96-well microtiter plates at a density of 4000 cells/well, grown in 5% CO_2 at 37°C for 24 h, and then exposed to different concentrations of target compounds (0.01–10 μmol/L), etoposide (0.01–100 μmol/L) and 0.5% DMSO (as control) for 48 hours. Read the OD values at 570 nm on a microplate reader (BIORAD Model 680, America) and get the inhibition ratio:

$$[\text{Inhibition ratio} = (\text{OD of control} - \text{OD of treatment})/(\text{OD of control} - \text{OD of blank}) \times 100] \quad (1)$$

Data were analyzed in Excel and the IC_{50} values (μmol/L) were determined graphically from cell survival curves. All assays were done in triplicate.

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