SUPPLEMENTARY MATERIAL

Synthesis, cytotoxicity and molecular docking of methylated (-)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin derivatives as novel antitumor agents

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Abstract: A series of novel methylated (-)-epigallocatechin-3-gallate-4β-triazolopodophyllotoxin derivatives is synthesized by utilizing the click reaction. Evaluation of their cytotoxicity against a panel of five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, SW480) using the MTT assay shows that most of these compounds exhibit weak cytotoxicity. It is observed that compound 12 shows the highest activity against A-549 cells with an IC50 value of 10.27 ± 0.90 μM. Molecular docking results suggested that this compound 12 has a higher binding affinity for EGFR than for tubulin. Our findings support the utility of compound 12 as a novel compound for the further development of anticancer agents.
**Keywords:** Podophyllotoxin; EGCG; click reaction; antitumor activity; molecular docking
Experimental

General information
Podophyllotoxin (PPT) and (−)-Epigallocatechin-3-gallate (EGCG) were obtained from Chengdu Proifa Technology Development Co., Ltd (Chengdu, China); 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were commercially available and used without further purification unless indicated otherwise. Melting points were measured by an X-4 melting point apparatus and were uncorrected. MS data were obtained in the ESI mode on API Qstar Pulsar instrument; HRMS data were obtained in the ESI mode on LCMS-IT-TOF (Shimadzu, Kyoto, Japan); \(^1\)H-NMR and \(^{13}\)C-NMR spectra were recorded on Bruker AVANCE III 500 MHz, or 600 MHz (Bruker BioSpin GmbH, Rheinstetten, Germany) instruments, using tetramethylsilane (TMS) as an internal standard: chemical shifts (\(\delta\)) are given in ppm, coupling constants (\(J\)) in Hz, the solvent signals were used as references (CDCl\(_3\): \(\delta_C = 77.2\) ppm; residual CHCl\(_3\) in CDCl\(_3\): \(\delta_H = 7.26\) ppm; CD\(_3\)OD: \(\delta_C = 49.0\) ppm; residual CH\(_3\)OH in CD\(_3\)OD: \(\delta_H = 4.78\) ppm). Column chromatography (CC): silica gel (200 – 300 mesh; Qingdao Makall Group CO., LTD; Qingdao; China). All reaction was monitored using thin-layer chromatography (TLC) on silica gel plates, which was visualized by ultraviolet light (254 nm) and/or 10% phosphomolybdic acid/EtOH.

Synthesis of Epipodophyllotoxin\(^{[1]}\)
To a solution of podophyllotoxin (0.4 g, 1 mmol) in dry CH\(_3\)CN (30 mL), NaI (0.5 g, 3 mmol) was added and stirred for 5 min. To this stirred suspension MeSO\(_3\)H (0.2 mL, 3 mmol) was added dropwise with syringe at 0 °C and the stirring was continued for another 5 h at room temperature. Nitrogen was bubbled through the solution to drive off the excess hydrogen iodide. This solution was then evaporated in vacuo and used for the next reaction without further purification. To the above crude product a mixture of H\(_2\)O-acetone (10 mL-10 mL) and anhydrous BaCO\(_3\) (0.4 g, 2 mmol) were added successively. After 30 min at 40 °C, the resultant mixture was diluted with methylene chloride (50 mL), then, poured into 10% NaS\(_2\)O\(_4\) solution (200 mL). The organic layer over Na\(_2\)SO\(_4\), concentrated in vacuo. Flash chromatography on silica gel with methylene chloride:acetone = 92:8 as eluent gave 0.4 g of epipodophyllotoxin.
(94%). $^1$H-NMR (CDCl$_3$, 400MHz) $\delta$ 6.92 (s, 1H, C$^5$-H), 6.45 (s, 1H, C$^8$-H), 6.33 (s, 2H, C$^2$, C$^6$-H), 5.93 (s, 2H, OCH$_2$O), 4.82 (d, 1H, $J = 3.4$ Hz, C$^4$-H), 4.57 (d, 1H, $J = 5.3$ Hz, C$^1$-H), 4.36–4.27 (m, 2H, C$^{11}$-CH$_3$), 3.69 (s, 3H, 4'-OCH$_3$), 3.69 (s, 6H, 3', 5'-OCH$_3$), 3.32–3.29 (m, 1H, C$^3$-H), 2.86–2.83 (m, 1H, C$^3$-H); $^{13}$C-NMR (CDCl$_3$, 100 MHz) $\delta$ 177.6 (C-12), 153.7 (C-3', C-5'), 149.5 (C-7), 148.6 (C-6), 138.0 (C-1'), 137.4 (C-9), 133.9 (C-4'), 133.0 (C-10), 110.8 (C-8), 110.7 (C-5), 109.4 (C-2', C-6'), 102.8 (OCH$_2$O), 69.4 (C-11), 67.1 (C-4), 61.0 (4'-OCH$_3$), 56.5 (3', 5'-OCH$_3$), 45.1 (C-1), 41.7 (C-2), 40.1 (C-3); MS-ESI m/z (%): 449 ([M+Cl]$^+$, 100).

Synthesis of 4'-Demethylepipodophyllotoxin$^{[2]}$

To a solution of podophyllotoxin (2.1 g, 5 mmol) in dry CH$_2$Cl$_2$ (50 mL), NaI (2.3 g, 15 mmol) was added and stirred for 5 min. To this stirred suspension MeSO$_3$H (1.5 g, 15 mmol) was added dropwise with syringe at 0 °C and the stirring was continued for another 5 h at room temperature. Nitrogen was bubbled through the solution to drive off the excess hydrogen iodide. This solution was then evaporated in vacuo and used for the next reaction without further purification. To the above crude product a mixture of H$_2$O-acetone (25 mL–25 mL) and anhydrous BaCO$_3$ (2.0 g, 10 mmol) were added successively. After 30 min at 40 °C, the resultant mixture was diluted with methylene chloride (100 mL), then poured into 10% NaS$_2$O$_4$ solution (500 mL). The organic layer over Na$_2$SO$_4$, concentrated in vacuo. Flash chromatography on silica gel with methylene chloride:acetone = 92:8 as eluent gave 1.8 g of 4'-Demethylepipodophyllotoxin (90%). $^1$H-NMR (CDCl$_3$, 400MHz) $\delta$ 7.12 (s, 1H, C$^8$-H), 6.94 (s, 1H, C$^5$-H), 6.33 (s, 2H, C$^2$, C$^6$-H), 5.93 (d, 2H, $J = 12.7$ Hz, OCH$_2$O), 4.58 (d, 1H, $J = 5.4$ Hz, C$^4$-H), 4.54 (d, 1H, $J = 5.2$ Hz, C$^1$-H), 4.29 (s, 1H, C$^{11}$-CH$_3$), 4.26 (d, 1H, $J = 8.2$ Hz, C$^{11}$-CH$_3$), 3.67 (s, 6H, 3', 5'-OCH$_3$), 3.31 (dd, 1H, $J = 10.2$ Hz, 17.3 Hz, C$^2$-H), 2.87 (m, 1H, C$^3$-H); $^{13}$C-NMR (CDCl$_3$, 100 MHz) $\delta$ 175.6 (C-12), 148.7 (C-7), 147.9 (C-3'), 147.9 (C-5'), 147.7 (C-6), 135.9 (C-1'), 134.2 (C-9), 133.0 (C-10), 131.5 (C-4'), 110.5 (C-8), 100.5 (C-5), 109.5 (C-2'), 109.5 (C-6'), 102.1 (OCH$_2$O), 68.3 (C-11), 66.7 (C-4), 56.6 (3', 5'-OCH$_3$), 44.6 (C-1), 41.1 (C-2), 39.4 (C-3); MS-ESI m/z (%): 423 ([M+Na]$^+$, 100).
Synthesis of 4β-azido-podophyllotoxins (10 and 11)

To a solution of epipodophyllotoxin/4'-Demethylepipodophyllotoxin (1.0 mmol) and sodium azide (5.0 mmol) in trichloromethane (4 mL) was added trifluoroacetic acid (TFA, 13.2 mmol) dropwise. The reaction mixture was stirred for 1 h, but in order to avoid gel formation during the reaction it was necessary to add further TFA (52.8 mmol). The solution was neutralized with aqueous saturated sodium bicarbonate (NaHCO₃). The phases were separated. The aqueous phase was extracted twice with CHCl₃ (20 mL). The combined organic phases were washed with water and dried over Na₂SO₄. Then the solvent was evaporation and the reaction mixture was chromatographed on silica gel to afford the product.²,³

4β-Azido-4-deoxypodophyllotoxin (10)

Yield 60 %. ¹H-NMR (CDCl₃, 400 MHz) δ 7.05 (s, 1H, C⁸-H), 6.63 (s, 1H, C⁵-H), 6.35 (s, 2H, C²', C⁶'-H), 6.05 (d, 2H, J = 3.6 Hz, OCH₂O), 5.11 (d, 1H, J = 3.5 Hz, C⁴-H), 4.65 (d, 1H, J = 3.5 Hz, C¹-H), 4.36 (dd, 1H, J = 8.3 Hz, 7.4 Hz, C¹¹-CH₂β), 4.22 (dd, 1H, J = 8.6 Hz, 10.2 Hz, C¹¹-CH₂α), 3.67 (s, 6H, 3', 5'-OCH₃), 3.66 (s, 3H, 4'-OCH₃), 3.21 (dd, 1H, J = 5.3 Hz, 14.0 Hz, C²-H), 3.12 (m, 1H, C³-H); ¹³C-NMR (CDCl₃, 100 MHz) δ 174.5 (C-12), 153.5 (C-3'), 153.5 (C-5'), 149.6 (C-7), 148.0 (C-6), 136.6 (C-1'), 136.6 (C-4'), 133.5 (C-9), 128.5 (C-10), 111.4 (C-5), 109.7 (C-8), 109.4 (C-2'), 109.4 (C-6'), 102.7 (OCH₂O), 68.2 (C-11), 66.2 (C-4), 56.3 (3', 5'-OCH₃), 44.5 (C-1), 41.6 (C-2), 37.8 (C-3); MS-ESI m/z (%): 462 ([M+Na]⁺, 100).

4β-Azido-4-deoxy-4'-demethypodophyllotoxin (11)

Yield 40%. ¹H-NMR (CDCl₃, 400MHz) δ 7.07 (s, 1H, C⁵-H), 6.60 (s, 1H, C⁸-H), 6.38 (s, 2H, C²', C⁶'-H), 6.05 (d, 2H, J = 0.6 Hz, OCH₂O), 4.61 (q, 2H, J = 3.7 Hz, 5.3 Hz, C⁴-H, C¹-H), 4.36 (dd, 2H, J = 8.5 Hz, 10.3 Hz, C¹¹-CH₂), 3.66 (s, 6H, C³', C⁵'-OCH₃), 3.11 (dd, 1H, J = 4.7 Hz, 14.1 Hz, C²'-H), 2.96 (m, 1H, C³-H); ¹³C-NMR (CDCl₃, 100 MHz) δ 174.3 (C-12), 148.9 (C-3'), 148.6 (C-5'), 148.0 (C-7), 148.0 (C-6), 136.0 (C-1'), 133.9 (C-4'), 131.2 (C-9), 129.4 (C-10), 110.8 (C-5), 109.2 (C-2'), 109.2 (C-6'), 107.5 (C-8), 102.6 (OCH₂O), 70.9 (C-11), 63.9 (C-4), 56.4 (C³', C⁵'-OCH₃), 45.7 (C-1), 44.4 (C-2), 38.6 (C-3); MS-ESI m/z (%): 448 ([M+Na]⁺, 100).
**Figure S1.** The similarity of ligand redocked the site of protein. **A.** The structures of tubulin (PDB ID: 3UT5) and ligands. **B.** The structures of EGFR (PDB ID: 3IKA) and ligands. The protein is shown as cartoon, ligands are shown as cyan sticks; redocked ligands are shown as yellow sticks.

**Figure S2.** Binding conformations of compound **12** at the active site of protein. **A.** Proposed binding modes of colchicine (cyan) and compound **12** (yellow) in the active site of tubulin (PDB ID: 3UT5). **B.** Proposed binding modes of WZ4002 (cyan) and compound **12** (yellow) in the active site of EGFR (PDB ID: 3IKA).
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Figure S3. $^1$H-NMR of epipodophyllotoxin.

Figure S4. $^{13}$C-NMR of epipodophyllotoxin.
Figure S5. $^1$H-NMR of 4'-Demethylepipodophyllotoxin.

Figure S6. $^{13}$C-NMR of 4'-Demethylepipodophyllotoxin.
Figure S7. $^1$H-NMR of 4β-azido-4-deoxypodophyllotoxin (10).

Figure S8. $^{13}$C-NMR of 4β-azido-4-deoxypodophyllotoxin (10).
Figure S9. $^1$H-NMR of 4β-azido-4-deoxy-4'-demethypodophyllotoxin (11).

Figure S10. $^{13}$C-NMR of 4β-azido-4-deoxy-4'-demethypodophyllotoxin (12).
Figure S11. $^1$H-NMR of compound 7.

Figure S12. $^{13}$C-NMR of compound 7.
Figure S13. $^1$H-NMR of compound 8.

Figure S14. $^{13}$C-NMR of compound 8.
Figure S15. $^1$H-NMR of compound 9.

Figure S16. $^{13}$C-NMR of compound 9.
Figure S17. $^1$H-NMR of compound 12.

Figure S18. $^{13}$C-NMR of compound 12.
Figure S19. $^1$H-NMR of compound 13.

Figure S20. $^{13}$C-NMR of compound 13.
Figure S21. $^1$H-NMR of compound 14.

Figure S22. $^{13}$C-NMR of compound 14.
Figure S23. $^1$H-NMR of compound 15.

Figure S24. $^{13}$C-NMR of compound 15.