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# Synthesis and cytotoxic activity of novel derivatives of 4′-demethylepipodophyllotoxin

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**Abstract**—Nine novel 4β-*N*-substituted-5-FU-4′-demethylepipodophyllotoxin derivatives were synthesized and evaluated as potential antitumor agents. All of the target compounds showed more significant cytotoxic activity against HL-60 and A-549 in vitro than VP-16 and 5-FU. Among them, 4β-*N*-substituted-phenylalanine 5-Fu pentyl ester-4′-demethylepipodophyllotoxin (**9g**) was found to exhibit most potent cytotoxic activity against HL-60 and A-549 cell ($IC_{50}$ is 0.04 and <0.01 μM, respectively).


Podophyllotoxin (**1**) and its derivatives exhibit pronounced biological activity as antiviral agents and antineoplastic drugs.[1,2] The podophyllotoxin derivatives etoposide (VP-16, **2a**), etopophos (etoposide phosphate, **2b**), and teniposide (VM-26, **2c**) are currently used in the chemotherapy for various types of cancer, including small cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma.[3–5] NK611 (**2d**), as well as GL331 and TOP53 are presently under clinical trials.[1,6] In addition, podophyllic aldehyde and its analogues were found to be a highly selectivity against the HT-29 colon carcinoma.[7,8] Interestingly, these semisynthetic derivatives and the parent compound, podophyllotoxin, showed different mechanisms of action.[9] Podophyllotoxin inhibits the assembly of tubulin into microtubules through interaction with protein at the colchicine binding site, preventing the formation of the spindle.[1,9] While etoposide and congeners induce a premitotic blockade in late S stage of the cell cycle because of the inhibition of DNA topoisomerase II(TopII), an enzyme required for the unwinding of DNA during replication. Etoposide binds to and stabilizes the DNA–protein complex preventing religation of the double-stranded breaks.[1,9] However, due to the typical adverse effects, such as anemia, hair loss and severe gastrointestinal disturbances, the application of them has been limited to a certain extent.[10] Therefore, being continued research on podophyllotoxin is currently focused on structure optimization to generate derivatives with superior pharmacological profiles and broader therapeutic scope.[2,11]

Antimetabolite 5-fluorourcial (5-FU, **3**) is one of the major anticancer agents used clinically for the treatment of stomach, colorectal, head, and neck cancers.[12] However, response rate and duration have limited its efficacy, although some 5-FU dosing schedules such as continuous infusion have increased the response rate compared to bolus and injection.[13] One main strategy of potentiating the antitumor activity of 5-FU is chemical and biochemical modulations by combination with cytotoxic or noncytotoxic agents (Fig. 1).[14]

As mechanism of action is concerned, 5-FU also belongs to DNA-interacting agent.[14] According to the principle of drug design, combination of 4′-demethylepipodophyllotoxin and 5-FU may overcome some faults and lead to decreased repair of DNA damage or increased DNA adduct formation in cancer cells. Structure–activity relationship (SAR) studies suggested that the structural features essential for the antineoplastic activity include 4′-demethylation, 4-epimerization, 4-substituted, and the *trans*-fused lactone ring.[15] And it was found that C4β-*N*-substituted (including 4β-aniline or alkylamino,[16–18] 4β-amido or sulphonamido[19] and amino acid[20,21]) congeners of podophyllotoxin resulted in inhibitors, which have biological activity comparable or superior to podophyllotoxin.

Based on the above, as well as amino acids with better biological activity and good water solubility in the human body, we combined 4′-demethylepipodophyllotoxin and

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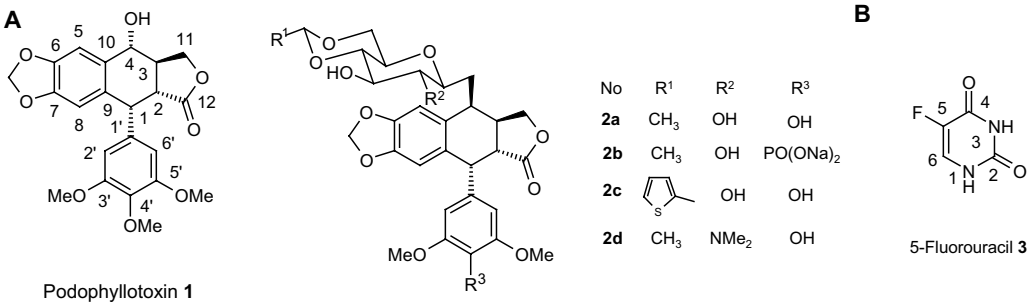
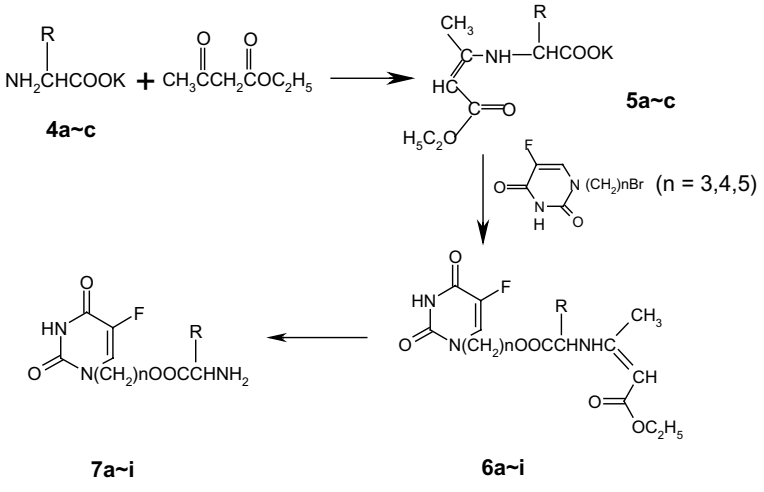



**Figure 1.** (**A**) Structure of podophyllotoxin and related compounds. (**B**) Structure of 5-fluorouracil.

5-FU with natural L-amino acid, and hoped to improve their anticancer activity and decrease their cytotoxicity for normal cells. Here, we report the synthesis of novel 4β-*N*-substituted-5-FU-4′-demethylepipodophyllotoxin and evaluation of cytotoxicity against human leukemic (HL-60) and human lung carcinoma (A-549) in vitro. The preliminary results showed these compounds possessed more significant cytotoxicity against HL-60 and A-549 than both VP-16 and 5-FU.

As shown in Scheme 1, compounds **7a**–**i** were synthesized in three steps from potassium salts of L-amino acids **4a**–**c**.[22] Compounds **4a**–**c** were treated initially with ethyl acetoacetate to afford **5a**–**c**, in which the α-amino group of amino acid were protected. N-protected L-α-amino acid 5-fluorouracil–propyl (butyl or pentyl) esters **6a**–**i** were prepared through combination **5a**–**c** with 1-[ω-bromo-propyl (butyl or pentyl)]-5-fluorouracil[23] under catalyzed by KI in *N*,*N*-dimethylformamide (DMF). Subsequent deprotection of compounds **6a**–**i** with methanolic HCl and basification with triethylamine in methylene chloride yielded L-α-amino acid 5-fluorouracil–propyl (butyl or pentyl) esters **7a**–**i**. As described in Scheme 2, 4β-*N*-substituted-amino acid 5-FU propyl (butyl or pentyl) ester—4′-demethylepipodophyllotoxins **9a**–**i**[24] were synthesized by direct nucleophilic substituted ($S_N1$) of 1.3 equivalent appropriate **7a**–**i** with 4β-bromo-4′-demethyl-epipodophyllotoxin **8**, which prepared according to literature,[25] in the presence of triethylamine in refluxing THF.

While synthesis of the target compounds, the bulky C-1 α pendant aromatic ring E directed the substitution to be stereoselective in yielding the C-4β alkylamino isomers as the main products. In some cases, C-4α isomers were also observed, but as minor products. The C-4β alkylamino isomers were purified by column chromatography. The assignment of the configuration at C-4 position for compounds **9a**–**i** was based on $J_{3,4}$ coupling constants. The C-4β substituted compounds have $J_{3,4} \approx 4.0$ Hz due to a *cis* relationship between H-3 and H-4. The C-4α substituted compounds, however, have

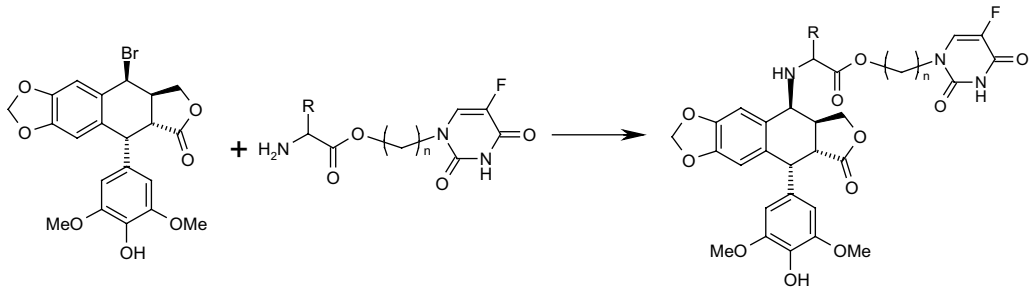



**4a, 5a** R=$CH_2Ph$
**4b, 5b** R=$CH(Me)CH_2Me$
**4c, 5c** R=$CH_2SMe$

compds **6, 7**

| n | R=$CH_2Ph$ | R=$CH(Me)CH_2Me$ | R=$CH_2SMe$ |
|---|---|---|---|
| 3 | **6a,7a** | **6b,7b** | **6c,7c** |
| 4 | **6d,7d** | **6e,7e** | **6f,7f** |
| 5 | **6g,7g** | **6h,7h** | **6i, 7i** |

**Scheme 1.** Synthesis of L-amino acid 5-fluorouracil esters **7a**–**i**.




**Scheme 2.** Synthesis of compounds **9a**–**i** (R and *n*, see the Table 1).

$J_{3,4} \geqslant 10.0$ Hz because of H-3 is *trans* to H-4.[26,27] The relative stereochemistry at C-4 position was also demonstrated by the observation of NOE between H-3 and H-4. While H-3 was irradiated in $^1$H NMR spectrum, the H-4 was enhanced 4–5%, however, H-2 was not enhanced. Thus we ensured that H-3 is *cis* to H-4 and H-2 is *trans* to H-3.

Cytotoxicities of all the target compounds against two tumor cell lines HL-60 and A-549 were tested in vitro following procedure of the methods described by Bergeron.[28] The results of these assays were used to obtain the corresponding inhibition rates, from which $IC_{50}$ (μM) values were calculated. As shown in Table 1, compounds **9a**–**i** are more active than both VP-16 and 5-FU. Compound **9g** is the most potent against HL-60 and A-549 cells.

In conclusion, the strategy to synthesize novel derivatives of podophyllotoxin through combination of antimetabolite 5-FU and TOP-II inhibitor 4′-demethylepipodophyllotoxin with L-amino acid may be successful. The target compounds **9a**–**i** showed more effective cytotoxic activity than both VP-16 and 5-FU. The $IC_{50}$ values of compounds **9a**–**i** against HL-60 and A-549 were less than those of VP-16 and 5-FU. Among them, Compound **9g** was the most potent cytotoxic agent with $IC_{50}$ values 0.04 μM against HL-60 and less than 0.01 μM against A-549, respectively. Further investigation of this series of compounds is underway.

**Table 1.** Cytotoxic activity of **9a**–**i** in vitro ($IC_{50}$, μM)

| Compd | *n* | R | HL-60[a] | A-549[b] |
|---|---|---|---|---|
| **9a** | 3 | $CH_2Ph$ | 0.31 | 0.48 |
| **9b** | 3 | $CH(Me)CH_2Me$ | 0.24 | 0.18 |
| **9c** | 3 | $CH_2SMe$ | 0.99 | 0.29 |
| **9d** | 4 | $CH_2Ph$ | 0.42 | 0.30 |
| **9e** | 4 | $CH(Me)CH_2Me$ | 0.53 | 0.74 |
| **9f** | 4 | $CH_2SMe$ | 0.05 | 1.87 |
| **9g** | 5 | $CH_2Ph$ | 0.04 | <0.01 |
| **9h** | 5 | $CH(Me)CH_2Me$ | 0.30 | 0.53 |
| **9i** | 5 | $CH_2SMe$ | 0.04 | 0.23 |
| **VP-16** | | | 2.75 | 7.38 |
| **5-FU** | | | 65.3 | 50.5 |

[a] MTT methods, drug exposure was for 48 h.
[b] SRB methods, drug exposure was for 72 h.

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24. To a solution of **8** (3 mmol) in 15 mL dry THF was added 30 mg triethylamine and a solution of **7** (10 mmol) in dry THF (5 mL). The mixture was refluxed under Ar. While TLC analysis showed that most of the starting material had been converted, the reaction was stopped. Then the mixture was filtered and evaporated under reduced pressure. The residue was purified by silica gel column chromatography to afford compound **9**. For example, 4β-*N*-substituted-phenylalanine 5-Fu-pentyl ester-4′-demethylepipodophyllotoxin **9g**: yield 23.8%; mp 125–127 °C; $[\alpha]_D^{25}$ −61 (*c* 0.3, acetone); IR (KBr) 3443, 3066, 2938, 2837, 1774, 1714, 1693, 1613, 1505, 1428, 1366, 1231, 1114 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300M) $\delta$ 9.64 (br, 1H, NH), 7.28–7.12 (m, 6H, H-6 of 5-Fu, $CH_2Ph$), 6.47 (s, 1H, H-5), 6.42 (s, 1H, H-8), 6.20 (s, 2H, H-2′, 6′), 5.93 (s, 2H, $OCH_2O$), 5.49 (br, 1H, 4′-OH), 4.43 (d, 1H, H-1, $J$ = 5.4 Hz), 4.27(s, 1H, OC*H*a$CH_2CH_2CH_2CH_2$5-FU), 4.24 (s, 1H, OC*H*b-$CH_2CH_2CH_2CH_2$5-FU), 4.06 (m, 2H, H-11), 3.97 (d, 1H, H-4, $J$ = 2.7 Hz), 3.72 (s, 6H, OMe-3′, 5′), 3.64 (t, 2H, C$H_2$-N-5Fu, $J$ = 7.2 Hz), 3.49 (t, 1H, C*H*$CH_2$Ph, $J$ = 6.0 Hz), 3.14 (dd, 1H, H-2, $J$ = 14.1, 5.7 Hz), 2.98 (d, 2H, C$H_2$Ph, $J$ = 6.0 Hz), 2.76 (m, 1H, H-3), 1.61 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$5-FU), 1.25 (m, 2H, $CH_2CH_2CH_2$-$CH_2CH_2$ 5-FU);[13] C NMR ($CDCl_3$, 75M) $\delta$ 175.4, 173.7, 157.3, 149.4, 147.7, 146.8, 146.2, 140.3, 136.6, 133.8, 131.9, 131.1, 130.9, 129.0, 128.8, 128.5, 126.9, 110.4, 108.5, 107.8, 101.3, 68.2, 64.4, 62.4, 56.3, 54.6, 48.6, 43.5, 40.6, 39.9, 38.6, 28.3, 27.9, 22.6; HRMS (ESI): 746.2705 for $[M+H]^+$ (calcd 746.2720 for $C_{39}H_{41}O_{11}N_3F$).
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