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Synthesis and Biological Evaluation of New 4β-5-Fu-substituted 4'-Demethylepipodophyllotoxin Derivatives

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Abstract: A series of new 4β-5-Fu-substituted 4'-demethylepipodophyllotoxin derivatives were synthesized and evaluated, together with some previously prepared ones, for their cytotoxic activities against four tumor cell lines (HL60, P388, A549 and BEL7402). Three of these compounds exhibited superior *in vitro* anticancer activity against P388 and A549 than the reference compound etoposide. In addition, the partition coefficients (*P*) of all the new and previously synthesized derivatives were determined.

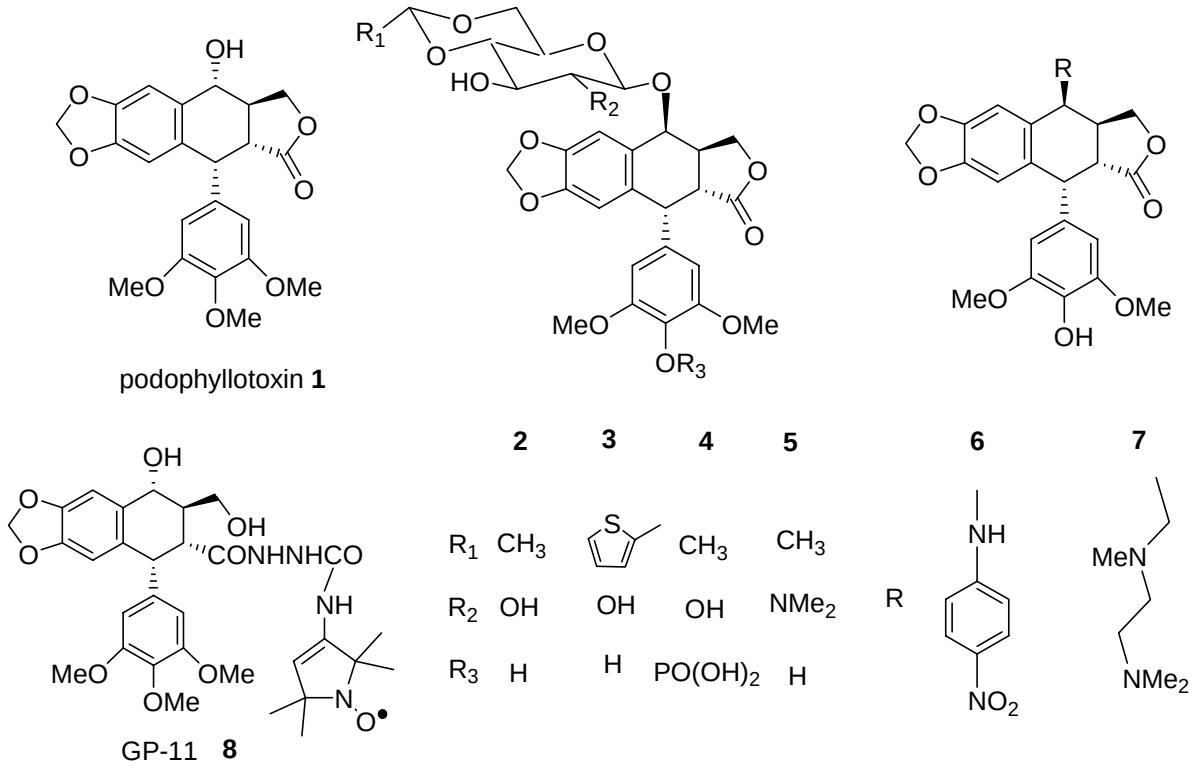
Keywords: 4'-Demethylepipodophyllotoxin, 5-fluorouracil, anticancer activities, partition coefficients

Introduction

Podophyllotoxin (**1,** Figure 1) exhibits high cytotoxic activity against various cancer cell lines, but its severe toxic side-effects have prevented it from being used directly as a therapeutic agent and this has prompted the search for derivatives with a greater therapeutic window [1,2]. Etoposide (VP-16, **2**), teniposide (VM-26, **3**) and etopophos (**4**) are three semisynthetic derivatives currently in clinical use as

antineoplastic agents [3]. Unfortunately, several drawbacks, such as myelosuppression, anemia, metabolic inactivation, development of drug resistance, severe gastrointestinal side effects, cytotoxicity towards normal cells and poor bioavailability, still exist during the administration of these drugs, so extensive structural modifications of podophyllotoxin at various positions have been undertaken in many laboratories to discover and develop more potent and less toxic anticancer agents [4-11], and some of these derivatives, such as NK-611 (**5**), GL-331 (**6**), TOP-53 (**7**), are currently being tested in phase I or II clinical trials for treatment of various cancers [12-14].

Figure 1. Podophyllotoxin and related compounds.



5-Fluorouracil (5-FU, **9**, Scheme 1), an important clinically useful anticancer drug, was first synthesized in 1957 [15]. Combination chemotherapy including 5-FU has been used extensively in the treatment of a wide range of solid tumors [16], but its negative effects, such as mucositis, nausea, vomiting and cardiotoxicity have often been observed. To tackle these problems, numerous modifications of the 5-FU structure have also been performed. The *N*-1 or/and *N*-3 substituted derivatives, in particular, have exhibited improved pharmacological and pharmacokinetic properties, including increased bioactivity, selectivity, metabolic stability, absorption and lower toxicity [17-22].

In recent years, nearly one hundred podophyllotoxin derivatives have been designed and synthesized and their biological activities against various cancers have been evaluated in the author's laboratory [23-28]. As a result some less toxic derivatives have been discovered, for example, GP-11 (**8**) [29]. As a part of our ongoing effort to find derivatives with improved anticancer activity and water solubility, we first designed a new series of derivatives seeking to combine the different anticancer mechanisms of 4'-demethyepipodophyllotoxin and 5-fluorouracil. In a previous communication [26], we reported the synthesis of seven novel derivatives and the evaluation of their activity against only

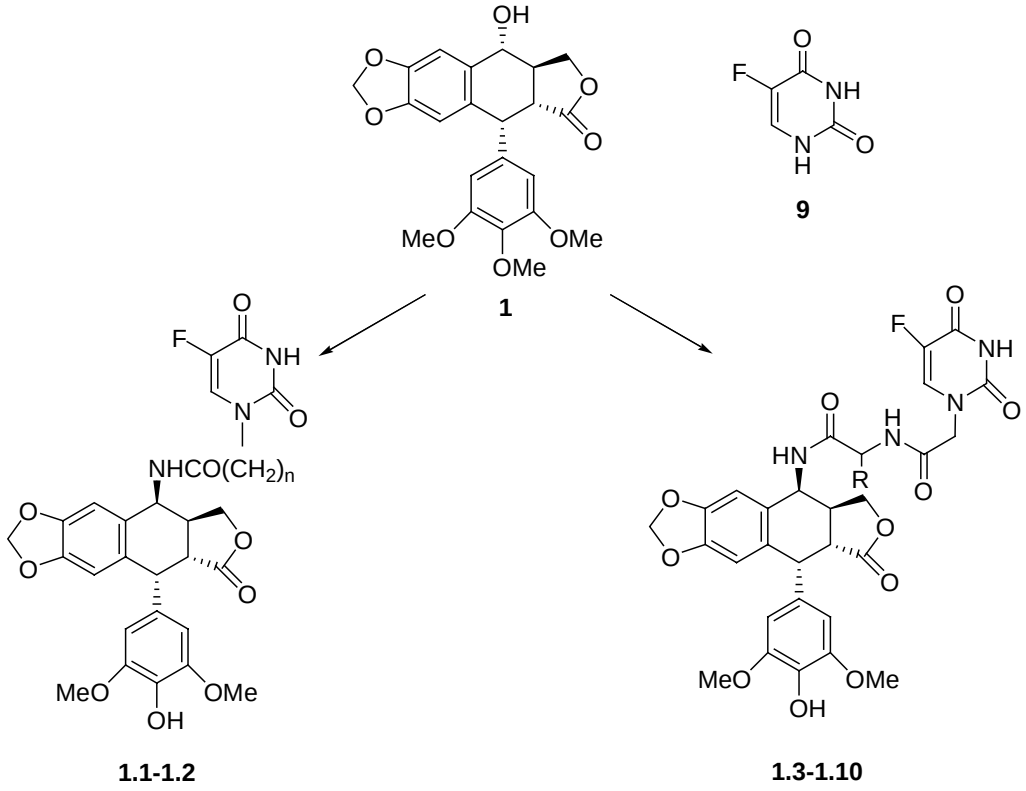
two matrix metalloproteinases (MMP1 and MMP3). Based on the above encouraging activity results, we report herein the synthesis of another three new derivatives, the anticancer activities of all ten derivatives against four cancer lines (HL60, P388, A549 and BEL7402) and the determination of their partition coefficients.

Results and Discussion

Design aims

Drug resistance is the most important challenge in cancer treatment research. Clinically, the cancer cells can't be totally "killed" by using a single drug for a long period of time, as they will become resistant to this drug and other drugs with a similar mechanism of action. In order to ensure efficacy, the optimal administration schedule involves a combination of two or more drugs, especially drugs with different mechanisms of action [30]. Based on the combination principle of drug design, the two drugs may be connected directly or by means of a linker. This technique can be used to overcome many problems including poor solubility, absorption, patient acceptability, instability and toxicity, and especially drug resistance. Natural *L*-amino acids are good pharmacophore carriers as well as good kinetophores, so we sought to combine demethyepipodophyllotoxin (an inhibitor of topoisomerase II) and 5-FU (a nucleoside antimetabolite) through a peptide bond derived from a natural *L*-amino acid.

Scheme 1. The synthesis [26] and structures of compounds **1.1**-**1.10.**



1.1 n = 1; **1.2** n = 2; **1.3** R = H; **1.4** R = CH_2CHMe_2; **1.5** R = $CHMeCH_2Me$; **1.6** R = CH_2CH_2SMe; **1.7** R = CH_2Ph; **1.8** R = Me; **1.9** R = $CHMe_2$; **1.10** R = CH(OH)Me.

At the same time, the significance of a drug's lipophilicity has widely been recognized by many researchers working in the drug discovery and drug design fields [31, 32]. The absorption, distribution, metabolism, excretion and toxicity of a drug are closely related with its lipophilicity, so this property must be considered in the rational design of anticancer drugs [33, 34]. The normal standard for expressing lipophilicity is the partition coefficient (*P*) in the immiscible *n*-octanol/water binary solvent system. Biologically active compounds, whose log *P* approaches zero either from the negative or positive side, should be ideal drugs, since these compounds possess appropriate hydrophilicity and lipophilicity. With this in mind our other aim in connecting these two types of anticancer drugs was to change demethyepipodophyllotoxin's hydrophilicity and 5-fluorouracil's lipophilicity, respectively, in the hope of producing derivatives that might be good drug candidates.

Chemistry

The three new compounds **1.8**-**1.10** were synthesized according to a previously published method [26] (Scheme 1).

Biological activity and partition coefficients

The cytotoxicities of compounds **1.1**-**1.10** were tested *in vitro* against four tumor cell lines (HL60, P388, A549 and BEL7402) [35, 36]. The assay results were then used to obtain the corresponding inhibition rates, from which IC_{50} values were calculated (Table 1). These synthetic 4′-demethyl-epipodophyllotoxin derivatives exhibited an interesting *in vitro* anticancer activity. Some of the derivatives were shown to be nearly equipotent or more potent than etoposide (**2**) and 5-Fu (**9**), two clinical drugs, in particular the compounds **1.8**, **1.9**, **1.10**, which exhibited highly potent *in vitro* anticancer activity against all four cancer cell lines.

Table 1. Cytotoxic Activity of **1.1**-**1.10** *in vitro* (IC_{50}, uM) and their partition coefficients (*P*).

Compounds	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**1.7**	**1.8**	**1.9**	**1.10**	**2**	**9**
HL-60 [a]	- [c]	- [c]	3.57	5.38	5.80	5.45	4.80	0.981	1.37	0.209	0.404	- [c]
P388 [a]	2.21	2.27	0.388	2.57	2.06	2.14	3.13	0.0473	0.0102	0.386	6.13	15.8
A549 [b]	3.56	10.5	1.74	2.85	3.25	3.74	4.07	0.036	0.522	0.0857	0.738	4.56
BEL7402 [b]	- [c]	- [c]	4.35	- [c]	- [c]	- [c]	- [c]	0.569	1.34	0.478	1.23	1.66
P	11.6	2.28	0.59	7.25	10.7	3.04	3.04	2.71	3.64	3.16	3.71	ND [d]

[a] MTT method, drug exposure was for 48h; [b] SRB method, drug exposure was for 72h.
[c] IC_{50} >100 uM. [d] ND: not determined.

The partition coefficients (*P*) of these derivatives were determined according to the published method [37], and the results are included in Table 1 (row five). The data showed that the logarithms of the partition coefficient of compounds **1.2**, **1.3**, **1.8** are all closer to zero than that of etoposide.

Structure-activity relationship (SAR) analysis

A simple structure-activity relationship (SAR) analysis was undertaken to study the influence of the different substituents on the cytotoxic potency of the synthesized compounds. The results can be summarized as follows: 1) compounds **1.1** and **1.2**, with no amino acid moiety, were less active than the other eight compounds, so we may conclude that the peptide bond is important for anticancer activity; 2) the relationship between the anticancer activities and the different substituents on the α-carbon of the amino acids appear to indicate that moderately sized hydrophobic substituents such as Me, $CHMe_2$ and CH(OH)Me increase the anticancer activity against HL60, A549 and P388, so in order to obtain anticancer derivatives with good activity it seems essential to consider such substituents at those positions; 3) for the anticancer activity it is also important to find a balance between the size of substituents and the partition coefficients.

Conclusions

In summary, we have synthesized some 4'-demethylepipodophyllotoxin derivatives that display more potent *in vitro* anti-cancer than the reference standard etoposide. Of special interest is the fact that we think that the activity of these new derivatives against four cancer cell lines may be synergistic. A study of the *in vivo* anticancer activities of compounds **1.8**, **1.9** and **1.10** is in progress.

Experimental

General

Melting points were determined on an X4 melting point apparatus and are uncorrected. The ^{1}H-NMR spectra were recorded in CD_3COCD_3 solutions containing TMS as an internal reference on a Bruker AM 400MHz spectrometer. IR spectra were recorded on a NIC-DX IR spectrometer. High-resolution mass spectra were recorded on a Bruker Daltonics APEX II 49e spectrometer using the ESI technique. Optical rotations were measured on a Perkin Elmer Model 341 digital polarimeter. Compounds **1.8, 1.9** and **1.10** were prepared according to the previously published method [26] and their analytical data are given below.

4'-O-Demethyl-4β-N-(5-FU acetyl-L-alanine acylamine)-4-desoxypodophyllotoxin (**1.8**). Yield: 83%; m.p. 192-194 ^{o}C; ^{1}H-NMR: 7.68 (d, *J* = 6.4 Hz, 5-FU ring H-6), 6.60 (s, 1H, H-5), 6.51 (s, 1H, H-8), 6.34 (s, 2H, H-2', 6'), 5.98 (d, *J* = 2.8 Hz, OCH_2O), 5.28 (d, *J* = 3.2 Hz, H-4), 4.52 (s, 2H, 5-Fu ring *N*-substituted CH_2), 4.48 (m, 1H, amino acid α-CH), 4.25 (m, 1H, H-11), 3.96 (m, 1H, H-11'), 3.67 (s, 6H, OCH_3), 3.10 (m, 1H, H-2), 3.03 (m, 1H, H-3), 1.26 (d, *J* = 7.2 Hz, 3H, CH_3). IR (KBr) υ cm^{-1}, 3383, 1773, 1513, 1482, 1232, 931; HRMS (FAB) $C_{30}H_{29}O_{11}N_4FNa$ $(M+Na)^+$: calcd. 663.1709, found 663.1723; $[\alpha]_D^{22}$ = -72.4 ° (c = 0.5, CH_3COCH_3).

4'-O-Demethyl-4β-N-(5-FU acetyl-L-valine acylamine)-4-desoxypodophyllotoxin (**1.9**). Yield: 84%; m.p. 214-216 ^{o}C; ^{1}H-NMR: 7.80 (d, *J* = 6.5 Hz, 1H, 5-Fu ring H-6), 6.74 (s, 1H, H-5), 6.50 (s, 1H, H-

8), 6.34 (s, 2H, H-2', 6'), 5.97 (d, *J* = 3.1 Hz, 2H, OCH_2O), 5.28 (d, *J* = 4.9 Hz, 1H, H-4), 4.57 (m, 1H, H-1), 4.52 (s, 2H, 5-FU ring *N*-substituted CH_2), 4.27 (t, *J* = 7.2 Hz, 1H, H-11), 4.23 (m, 1H, amino acid α-CH), 3.85 (m, 1H, H-11'), 3.67 (s, 6H, OCH_3), 3.13 (dd, 1H, *J* = 4.9, 14.0 Hz, H-2), 3.05 (m, 1H, H-3), 2.13 (m, 1H, CH $(Me)_2$), 0.95 (m, 6H, CH_3); IR (KBr) υ cm^{-1}, 3200-3500, 1771, 1618, 1515, 1481, 1232, 929; HRMS (FAB) $C_{32}H_{33}O_{11}N_4FNa$ $(M+Na)^+$: calcd. 691.2016, found 691.2014; $[\alpha]_D^{22}$ = -49.5° (c = 0.5, CH_3COCH_3).

4'-O-Demethyl-4β-N-(5-FU acetyl-L-threonine acylamine)-4-desoxypodophyllotoxin (**1.10**). Yield: 73 %; m.p. 215-217 °C; ^{1}H-NMR: 7.79 (d, *J* = 6.5 Hz, 1H, 5-FU ring H-6), 6.76 (s, 1H, H-5), 6.50 (s, 1H, H-8), 6.35 (s, 2H, H-2', H-6'), 5.96 (s, 2H, H-13, H-13'), 5.27 (m, 1H, H-4), 4.56 (m, 1H, H-1), 4.52 (2H, 5-FU ring *N*-substituted CH_2), 4.47 (m, 1H, amino acid α-CH), 4.36 (m, 1H, CH (OH) Me), 4.22 (m, 1H, H-11), 3.92 (m, 1H, H-11'), 3.67 (s, 6H, OCH_3), 3.13 (m, 1H, H-2), 3.00 (m, 1H, H-3), 1.16 (d, *J* = 6.1 Hz, 3H, CH_3); IR (KBr) υ cm^{-1} 3530, 3389, 1773, 1513, 1483, 1231, 931; HRMS (FAB) $C_{31}H_{35}O_{12}N_5F$ $(M+NH_4)^+$ calcd. 688.2261, found 668.2264; $[\alpha]_D^{22}$ = -59.6 °(c = 0.5, CH_3COCH_3).

Biological evaluation: Cell growth inhibition assay

Four tumor cell lines (human promyelocytic leukemia HL60, murine lymphocytic leukemia P388, human non-small cell lung A549 and human hepatocarcinoma BEL7402 cancer) were obtained from the CGMCC, Shanghai, China. The cells were cultured in a humidified atmosphere (37 °C, 5% CO_2) in RPMI1640 medium supplemented with 10% FCS.

MTT method: The MTT colorimetric assay was used to determine growth inhibition for HL60 and P388 cancer cells [35]. Cells were plated in 96-well plates and allowed to attach for 24 h. The epipodophyllotoxin derivatives were dissolved in DMSO at 10 mM concentrations. Cells were exposed in triplicate wells to these derivatives at various concentrations for 48 h. After 48 h, the media was aspirated and MTT solution (diluted in serum free media, 1 mg/mL, 10 μL) was added to each well. After 4 h of incubation, the solution was centrifuged for 10 min under 2000 rpm limpid solution, extracted carefully with DMSO (200 μL), then shaken by oscillator. The absorbance at A550 nm was determined on a microplate reader (Bio-Rad Model 3350, Japan). IC_{50} values were determined from log plots of percent of control *vs.* concentration.

SRB method: The number of cells was determined by the sulforhodamine B method for A549 and BEL7402 cancer cells [36]. Cells were plated in 96-well plates and allowed to attach for 24 h. Epipodophyllotoxin derivatives were dissolved in DMSO at 10 mM concentrations. Cells were exposed in triplicate wells to these derivatives at various concentrations for 72 h. After removing the medium, the cultures were fixed to the plastic substratum by gently adding a 10% trichloroacetic acid (TCA) in 0.9% NaCl solution (0.1 mL). The cultures were incubated for 1 hour at 4 °C, and then washed five times with water to remove the TCA. Plates were air-dried and then stained for 30 minutes with 0.4% SRB in 1.0% acetic acid. The SRB was then removed and the cultures were quickly rinsed five times with 1.0% acetic acid to remove unbound dye. The cultures were air-dried and the bound

dye was solubilized with 10 mmol/L unbuffered Tris base (pH 10.5, 2 mL). Absorbance was read at 515 nm using a microplate reader (Bio-Rad Model 3350, Japan).

Determination of partition coefficients

The partition coefficients of compounds **1.1**-**1.10** were determined according to the published method [37]. Octanol and water were mutually saturated by vibration, and the two phases were separated by centrifugation. Stock solutions of compounds **1.1**-**1.10** were prepared using the water-saturated octanol, then mixed with the other phase and the two-phase mixtures were intensely stirred for 10 h. After separation of the phases by centrifugation, the absorbance of the aqueous solution and the octanol phase were measured with a UV spectrophotometer. The *P* values were calculated as follows: *P* = compound in octanol / compound in water. The reported *P* values are an average of four measurements.

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Sample Availability: Samples of the compounds mentioned are available from the authors.