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Synthesis and biological evaluation of novel conjugates of podophyllotoxin and 5-FU as antineoplastic agents

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ABSTRACT

A series of novel conjugates of podophyllotoxin and 5-FU were designed using association strategy and were synthesized by coupling 4′-demethylepipodophyllotoxin with 5-FU-N^1-alkyl amino acid ester. These derivatives have been evaluated for cytotoxicity in vitro against tumour cell lines (HL-60, K562, A-549 and AGS), and their octanol–water partition coefficients (log*P*) were also determined. As compared with VP-16, most compounds showed superior water solubility, as well as more potent inhibitions against these four tumour cell lines. Compound **21** showed interaction with calf thymus DNA, and it was relatively resistant to metabolism by human plasma.

1. Introduction

Anticancer drugs are rarely used singly to treat cancer, because only a few tumours are sensitive enough to be cured by single drugs. For a specific type of tumour, effective chemotherapy usually depends on suitable combinations.[1] Many combinations, which were named mutual drug, in clinical use consist of an antimetabolite with one or more other anticancer agents. A mutual drug usually consists of two different synergistic drugs joined together. The two drugs may be connected directly or by means of a linker.[2]

Podophyllotoxin (**1**) is an antimitotic natural product. Its inhibitory activity on cell growth led to the development of the clinically valuable anticancer agents, etoposide (**2**), teniposide (**3**) and the water-soluble prodrug, etoposide phosphate (**4**) (Fig. 1). The cytotoxic mechanism of these drugs is the inhibition of topoisomerase II, unlike the lead compound that inhibits mitosis.[3,4] Through extensive structure–activity relationship studies, several potential drug candidates were synthesized such as GL-331,[5] TOP-53,[6] NK611[7] and F11782.[8] Recently, more complex and diverse analogues have been synthesized either to get more potent compounds or to overcome drug resistance.[9] 4-N-substituted 4′-demethylepipodophyllotoxin derivatives, such as 4β-sulfonamide,[10] (4″-benzamide)-amino[11] and carbamate,[12] showed stronger inhibitions to tumour or to TOP-II. In addition, podophyllic aldehyde and its analogues were found to be of a high selectivity against the HT-29 colon carcinoma.[13] Furthermore, a number of multidrug approaches have been tried to enhance the tumour selectivity or to increase the aqueous solubility.[14]

Our group has been aiming at the discovery of new antitumour drugs of podophyllotoxin derivatives with improved bioactivities, lower toxicity and without resistance effects in recent years.[15,16] In the present work, we reported the synthesis of a series of conjugates of **1** together with 5-FU, in which the main chemical differences are the length of the side chain (propyl, butyl or pentyl), and the difference of amino acids. Their cytotoxic activities against four tumour cells (HL-60, K562, A-549 and AGS) were evaluated. Also, the octanol–water partition coefficients (log*P*) were measured. Furthermore, metabolism stability and interaction with calf thymus DNA (CT DNA) of **21** in vitro were studied.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds $\mathbf{7}_{\mathbf{I–III}}$ and **8** is outlined in Scheme 1. 5-FU was protected with 1,1,1,3,3,3-hexamethyldisilazane (HMDS)/chlorotrimethylsilane (TMSCl) and **6** was obtained in excellent yield; then **6** reacted with 1,3-dibromo-propane (or 1,4-dibromo-butane, or 1,5-dibromo-pentane) under 105–110 °C

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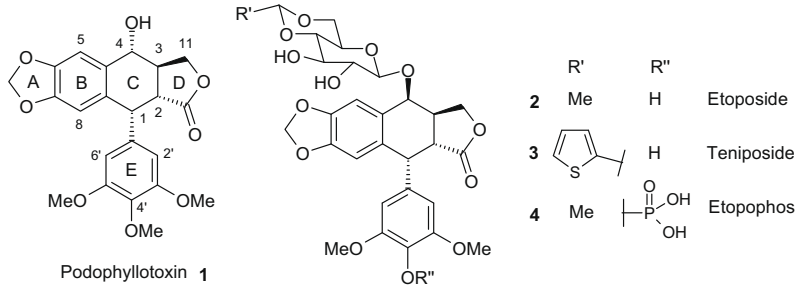


Figure 1. Podophyllotoxin (**1**), etoposide (**2**), teniposide (**3**) and etopophos (**4**).

resulting in the $\mathbf{7}_{I–III}$.[17] Compounds $\mathbf{8}_{Ia–h}$, $\mathbf{8}_{IId,g}$ and $\mathbf{8}_{IIId,g}$ were prepared in four steps from potassium salts of L-α-amino acids as in the previously reported method.[16,18]

Compounds **10**–**21** were synthesized from podophyllotoxin (**1**) according to the literature methods (Scheme 2).[19] Briefly, intermediate 4β-iodide 4′-demethylepipodophyllotoxin (**9**) was prepared stereoselectively from **1** through successive 4′-demethylation, 4-iodination with methanesulfonic acid/sodium iodide, and nucleophilic substitution with **8**, afforded target compounds **10**–**21** by one-pot reaction in the presence of triethylamine in refluxing THF. The structure of all target compounds was identified by ^{1}H NMR, ^{13}C NMR, IR and HRMS.

Biological activities of podophyllotoxin derivatives were affected heavily by their configurations at positions C-4 and C-2, compounds with the structure of C-4β substitute and *trans* lactone C-ring generally show more potent biological activities. So it is important to corroborate the relative configurations at C-4 and C-2 of target compounds. While synthesis of the target compounds **10**–**21,** the bulky C-1 α pendant aromatic ring E directed the substitute to be stereoselective in yielding the C-4β alkylamino isomers as the major products. In some cases, C-4α-isomers were also observed, but as minor products. The assignment of the configuration at C-4 position for target compounds was based on $J_{3,4}$ coupling constants[20] or 1D NOE. When proton of C-4 was irradiated in ^{1}H NMR spectrum for **21**, protons of C-3 and C-2′,6′ were enhanced by 5.13% and 5.12%, respectively; however, proton of C-2 was not enhanced. In addition, proton of C-2 was enhanced by 4.56% while proton of C-1 was irradiated for **21**, but protons of C-3 and C-4 were not enhanced. Thus, we confirmed that H-4 is *cis* to H-3 and H-2 is *trans* to H-3 for **21**.

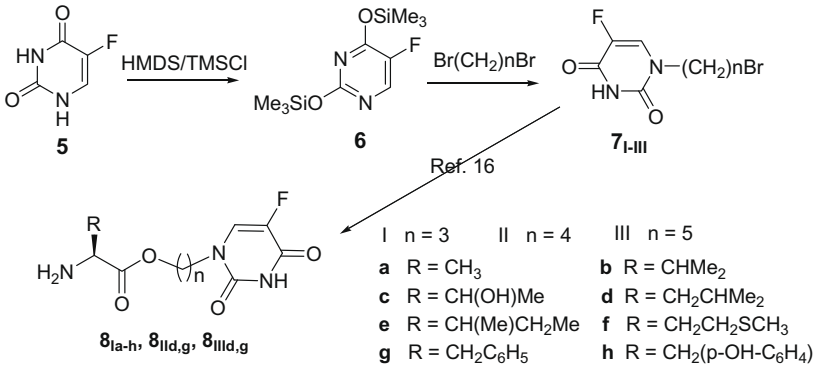
2.2. Biological activities

The biological activities of this series of podophyllotoxin derivatives were evaluated by an in vitro cytotoxicity test, which was carried out with a panel of four tumour cell lines that comprise human premyelocytic leukemia (HL-60), human erythromyeloblastoid leukemia (K562), human gastric cancer (AGS) and human lung carcinoma (A-549), using VP-16 (**1**) and 5-FU (**5**) as reference compounds. The screening procedure was based on the standard MTT or SRB method. The IC_{50} values are shown in Table 1.

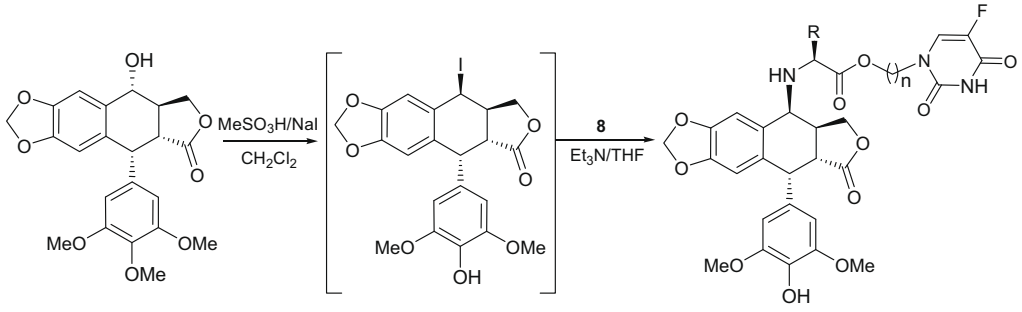
As shown in Table 1, **10**–**21** showed a basically stronger inhibition of these four cell lines than both VP-16 and 5-FU. In these four tumour cell lines, the inhibition of these target compounds showed decreased activity in the following: HL-60 ≈ A-549 > K562 > AGS. The large activity range of compounds **10**–**17** indicated that the substituent of the amino acids markedly affected the activity profiles of this compound class, and some important SAR information was revealed. As seen with compounds **12** and **17**, inclusion of moieties containing hydroxyl groups in the amino acid side chains decreased the activity significantly. This decrease may be caused by hydrogen bonding between the nitrogen or oxygen atoms and the enzyme/DNA might cause the loss of activity, or the relatively polar moieties might impede an important hydrophobic interaction between the molecules and topo II/DNA. The different lengths of alkyl linkages of target compounds did not affect obviously their cytotoxicities (**13** vs **18** vs **20**, **16** vs **19** vs **21**).

The octanol–water partition coefficients of **10**–**21** were also determined and results are shown in Table 1. The log*P* of **10**–**21** is lower than VP-16, which means that their water solubility is better than that of VP-16.

Ordinary, the analogues of **2** were considered as inhibitors of TOP-II; however, other compounds, which showed potent inhibitions against tumour cell lines, but not inhibitions to TOP-II were also found.[21] In order to study the interaction of **21** with CT DNA in pH 7.2 PBS, the CD spectrum of CT DNA and its complexes with **21** at various concentrations: 0 (1), 10 (2), 20 (3) and 50 μg/mL (4) were recorded from 200 to 320 nm at 5 °C on a Jasco-810 spectrometer, and the CD spectra of CT DNA in the presence and absence of **21** in pH 7.2 PBS are shown in Figure 2. The CD



Scheme 1. Synthesis of **7** and **8**.



1 **9** **10-21**

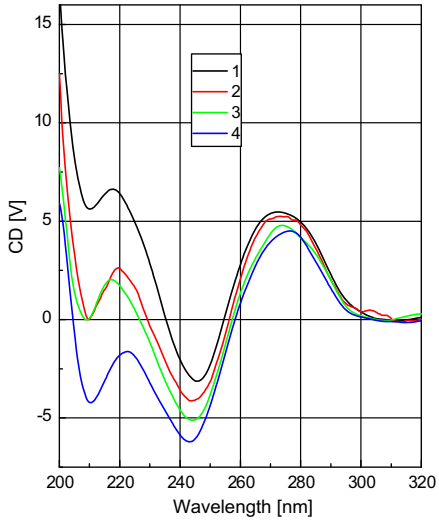
Compd	n	R	Compd	n	R	Compd	n	R
10	3	Me	**14**	3	$CH(Me)CH_2Me$	**18**	4	CH_2CHMe_2
11	3	$CHMe_2$	**15**	3	CH_2CH_2SMe	**19**	4	$CH_2C_6H_5$
12	3	CH(OH)Me	**16**	3	$CH_2C_6H_5$	**20**	5	CH_2CHMe_2
13	3	CH_2CHMe_2	**17**	3	$CH_2(p\text{-}OH\text{-}C_6H_4)$	**21**	5	$CH_2C_6H_5$

Scheme 2. Synthesis of compounds **10**–**21**.

Table 1
Biological evaluation of compounds **10**–**21**

Compound	Cytotoxic activities (IC_{50}, μM)[a]				log *P*
	K562[b]	AGS[c]	HL-60[b]	A-549[c]	
10	19.5	40.4	22.3	2.59	−0.07
11	13.5	62.5	5.80	1.92	0.34
12	30.8	140.8	23.2	6.95	−0.12
13	9.5	59.2	0.13	0.01	0.33
14	5.1	56.2	0.24	0.18	0.59
15	8.8	24.8	0.99	0.29	0.23
16	5.8	23.6	0.31	0.48	0.65
17	28.6	114.7	26.6	21.7	0.07
18	5.3	34.5	0.73	<0.01	0.35
19	9.2	23.7	0.42	0.30	0.43
20	6.0	23.5	0.31	0.53	0.51
21	13.2	38.8	0.04	<0.01	0.29
5-FU	>100	>100	65.3	50.5	—
VP-16	>100	50.8	2.75	7.38	0.69

[a] The value is the average of triplicate.
[b] MTT methods, drugs exposure was for 48 h.
[c] SRB methods, drugs exposure was for 72 h.



1. 25μg/mL Calf Thymus DNA 2. 1+10μg/mL **21**
3. 1+20μg/mL **21** 4. 1+50μg/mL **21**

Figure 2. CD spectra of calf thymus DNA.

spectrum of free CT DNA exhibits one positive peak at 272 nm due to the base stacking and one negative band at 245 nm due to the helicity, which is the characteristic of DNA in the right-handed B form (Fig. 2, curve 1).[22] Increasing the concentration of **21** (c = 10, 20, 50 μg/mL, respectively) in above CT DNA solution, the intensity of positive band decreases and negative band increases, as well as a gradual blue shift of the negative band from 245 to 243 nm and a red shift from 272 to 276 nm of the positive band were also observed (Fig. 2, curves 2, 3 and 4, respectively), suggesting that DNA bound with **21** induces certain conformational changes, DNA double helix in solution is in a more helical state belonging to C form DNA.[23] Double helix form DNA is not really a rigid molecule, especially in solution, and water molecules are important to the conformations of DNA. The major and minor grooves are 'coated' by a unimolecular layer of water molecules which interact with the exposed C=O, N and NH functions of ribose and also extensively solvate the phosphate backbone.[24] While the **21** binds to the DNA, it also interacts with the ribose and phosphate. Competition between water molecules and **21** induced water contents may be decreased in the micro surrounding around the DNA. This would make the helicity of DNA become stronger. Thus, the conformation of DNA is changed from the B-form to a more compact C-form. These changes of the CD spectrum of CT DNA revealed the interaction of CT DNA with **21**.

Further, metabolism stabilities of **21** were also investigated in human plasma, mouse plasma and pH 7.4 PBS (1.0 μM) within 24 h at 37 °C, respectively. As shown in Figure 3, **21** was stable within 24 h in human plasma and within 8 h in pH 7.4 PBS; however, its content was decreased dramatically in pH 7.4 PBS after 8 h, as well as in mouse plasma after 2 h, respectively. These re-

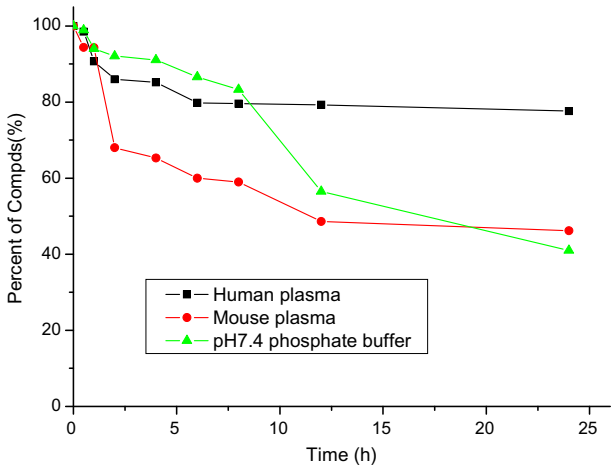


Figure 3. The stability of compound **21**.

sults revealed the following order of stability: human plasma > pH 7.4 phosphate buffer > mouse plasma.

3. Conclusion

In summary, we believed that conjugate of podophyllotoxin and 5-FU is a promising direction in antineoplastic agents finding, because the target compounds showed more effective cytotoxicities in vitro and superior water solubility than those of the reference etoposide. In addition, the conformation of DNA is changed from the B-form to the C-form in the presence of **21**, and the result revealed the interaction of CT DNA with **21**. Furthermore, compound **21** is stable in human plasma, but not enough stable in mouse plasma and pH 7.4 phosphate buffer solution.

4. Experimental

4.1. Chemistry. General

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. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury-300BB spectrometer with TMS as an internal standard, all chemical shift values are reported as δ ppm. Optical rotations were measured on a Perkin Elmer 341 polarimeter in a 1 dm cell at 23 °C. Mass spectra were recorded on a Bruker Daltonics APEXII49e and VGZAB-HS (70 eV) spectrometer with ESI and FAB source as ionization, respectively. HPLC analyses were carried out on a Shimadzu LC-10AT series HPLC system with SPD-M10AVP UV–vis detector. Circular dichroism spectra were measured using a JASCO-810 spectrometer. All reactions were monitored by thin layer chromatography (TLC) on silica gel GF_{254} (0.25 mm thick). CC was performed on Silica Gel 60 (230–400 mesh, Qingdao Ocean Chemical Ltd., China). Podophyllotoxin was isolated from a Chinese medicinal herb *Podophyllum emodi* Wall var Chinesis Sprague, other starting materials and regents were purchased commercially and used without further purified, unless otherwise stated.

4.2. General procedure of synthesis of 7

To dried 1, ω-dibromo alkane (20 mL) was added 2, 4-ditrimethylsilyl 5-FU (5.5 g, 20 mmol), followed by the mixture that was stirred for 3 h at 105 °C. The residue was cooled and diluted with $CHCl_3$ (20 mL), washed with water (2 × 15 mL), the aqueous layers were extracted with $CHCl_3$ (20 mL). The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4, filtered and concentrated to obtain yellow oil. Purification by recyclization with isopropanol afforded $\mathbf{7}_{I-III}$.

4.2.1. 1-(ω-Bromopropyl)-5-FU (7_I)

The yield 57%; mp: 112–113 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.81 (br, 1H, NH), 7.35 (d, J = 5.4 Hz, 1H, CH), 3.89 (t, J = 6.6 Hz, 2H, CH_2), 3.44 (t, J = 6.0 Hz, 2H, CH_2), 2.29–2.25 (m, 2H, CH_2). MS (EI): 252 ($[M+2]^+$), 250 ($[M]^+$), 185, 171 ($[M-Br]^+$).

4.2.2. 1-(ω-Bromobutyl)-5-FU (7_{II})

The yield 63%; mp: 129–130 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.81 (br, 1H, NH), 7.26 (d, J = 5.4 Hz, 1H, CH), 3.75 (t, J = 6.8 Hz, 2H, CH_2), 3.43 (t, J = 6.3 Hz, 2H, CH_2), 1.89–1.86 (m, 4H, $2CH_2$). MS (EI): 266 ($[M+2]^+$), 264 ($[M]^+$), 185 ($[M-Br]^+$).

4.2.3. 1-(ω-Bromopentyl)-5-FU (7_{III})

Yield 72%; mp: 106–108 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.49 (br, 1H, NH), 7.25 (d, J = 6.3 Hz, 1H, CH), 3.73 (t, J = 7.5 Hz, 2H, CH_2), 3.41 (t, J = 6.3 Hz, 2H, CH_2), 1.91–1.87 (m, 2H, CH_2), 1.73–1.69 (m, 2H, CH_2), 1.52–1.48 (m, 2H, CH_2). MS (EI): 280 ($[M+2]^+$), 278 ($[M]^+$), 199 ($[M-Br]^+$).

4.3. General preparation of compounds 10–21

To a solution of podophyllotoxin **1** (414 mg, 1 mmol) in dry CH_2Cl_2 (10 mL), NaI (447 mg, 3 mmol) was added and stirred for 5 min. To this stirred suspension, $MeSO_3H$ (288 mg, 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 intermediate **9** dissolved in dry THF (15 mL) was added the appropriate arylamine **8** (3 mmol), followed by triethylamine (30 mg, 1.1 mmol). The mixture was refluxed under nitrogen. While TLC analysis showed that most of the starting material had been converted, the reaction was stopped. The reaction mixture was diluted with ethyl acetate and washed with water, 10% $Na_2S_2O_4$ solution, dried and purified via CC to afford compounds **10**–**21**.

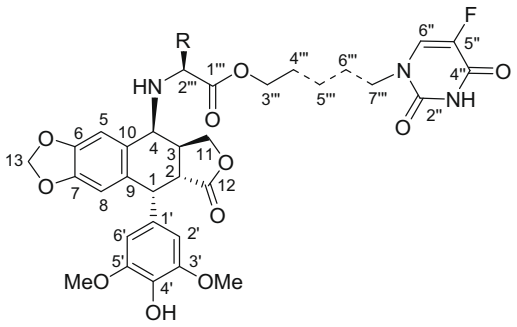
4.3.1. 4′-*O*-Demethyl-4β-*N*-[(5-FU-*N*^{1}-)-propyl L-alanyl]-4-desoxy-podophyllotoxin (10)

The yield 46%; mp: 122–124 °C; $[\alpha]_D^{23}$ −66 (*c* 0.3, acetone); IR (KBr) 3436, 3229, 3070, 2915, 1772, 1698, 1613, 1482, 1371, 1231, 1114 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28 (d, J = 5.4 Hz, 1H, H-6″), 6.69 (s, 1H, H-5), 6.47 (s, 1H, H-8), 6.24 (s, 2H, H-2′,6′), 5.95 (s, 1H, H-13a), 5.94 (s, 1H, H-13b), 4.51 (d, J = 5.4 Hz, 1H, H-1), 4.36–4.26 (m, 2H, H-3‴), 4.20 (t, J = 6.0 Hz, 2H, H-11), 3.98 (d, J = 3.9 Hz, 1H, H-4), 3.78 (t, J = 6.6 Hz, 2H, H-7‴), 3.74 (s, 6H, 3′,5′-OMe), 3.42 (q, J = 7.2 Hz, 1H, H-2‴), 3.30 (dd, J = 14.1, 5.4 Hz, 1H, H-2), 2.86–2.74 (m, 1H, H-3), 2.12–2.01 (m, 2H, H-4‴), 1.41 (d, J = 6.3 Hz, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 642.2095 for $[M+H]^+$ (calcd 642.2094 for $C_{31}H_{33}FN_3O_{11}$).

4.3.2. 4′-*O*-Demethyl-4β-*N*-[(5-FU-*N*^{1}-)-propyl L-valinyl]-4-desoxy-podophyllotoxin (11)

The yield 43%; mp: 122–124 °C; $[\alpha]_D^{23}$ −69 (*c* 0.3, acetone); IR (KBr) 3433, 2962, 1773, 1711, 1660, 1613, 1482, 1369, 1231, 1114 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29 (d, J = 5.4 Hz, 1H, H-6″), 6.67 (s, 1H, H-5), 6.47 (s, 1H, H-8), 6.23 (s, 2H, H-2′,6′), 5.95 (s, 2H, H-13), 5.50 (br, 1H, NH), 4.51 (d, J = 6.0 Hz, 1H, H-1), 4.32–4.28 (m, 2H, H-3‴), 4.23 (t, J = 5.7 Hz, 1H, H-11a), 4.18 (t, J = 6.0 Hz, 1H, H-11b), 3.96 (d, J = 3.6 Hz, 1H, H-4), 3.79 (t, J = 8.1 Hz, 2H, H-7‴), 3.74 (s, 6H, 3′,5′-OMe), 3.38 (dd, J = 13.5,

Table 2
^{13}C NMR data of compounds **10**–**21**



Compound	10	11	12	13	14	15	16	17	18	19	20	21
C-1	43.8	43.6	43.8	43.6	43.8	43.6	43.5	43.7	43.6	43.5	43.6	43.5
C-2	41.2	40.8	40.5	40.7	40.8	40.8	40.6	40.8	40.8	40.6	40.8	40.8
C-3	38.7	38.5	38.7	38.5	38.5	38.6	38.6	38.9	38.6	38.6	38.6	38.6
C-4	54.0	53.9	53.7	53.8	53.8	53.8	54.2	54.5	54.2	54.4	54.2	54.6
C-5	108.7	108.6	109.1	108.5	108.8	108.6	108.5	109.1	108.6	108.5	108.5	108.5
C-6	147.3	146.8	146.6	146.7	146.7	146.7	146.8	146.9	146.9	146.8	146.5	146.8
C-7	147.9	147.7	147.7	147.6	147.7	147.7	147.7	147.6	147.7	147.7	147.7	147.7
C-8	110.7	110.7	110.9	110.6	110.9	110.6	110.6	110.2	110.7	110.4	110.8	110.4
C-9	131.9	131.9	132.3	132.1	132.2	132.1	132.1	131.5	132.1	132.0	132.1	131.9
C-10	134.0	133.8	133.9	133.8	134.0	133.8	133.9	135.0	133.9	133.9	133.8	133.8
C-11	68.6	68.1	68.0	68.1	68.1	68.1	68.2	68.6	68.1	68.1	68.2	68.2
C-12	174.5	174.5	173.4	174.4	173.9	173.8	173.6	173.9	174.5	173.7	174.4	173.7
C-13	101.6	101.4	101.3	101.3	101.3	101.3	101.3	101.6	101.4	101.3	101.4	101.3
C-1′	131.4	131.2	131.5	131.2	131.4	131.2	131.2	130.5	131.5	131.1	131.2	131.1
C-2′,6′	108.1	107.9	107.9	107.8	108.0	107.9	107.9	108.8	107.9	107.9	107.8	107.8
C-3′,5′	146.5	146.2	146.3	146.2	146.3	146.3	146.3	147.2	147.2	146.2	146.3	146.2
C-4′	136.6	136.8	136.5	136.7	136.4	136.6	136.6	136.3	136.5	136.6	135.6	136.6
3′,5′-OMe	56.6	56.4	56.3	56.3	56.4	56.3	56.3	55.9	56.4	56.3	56.3	56.3
C-2″	149.8	149.3	149.6	149.4	149.3	149.5	149.3	149.9	149.3	149.4	149.4	149.4
C-4″	157.5	156.8	157.3	157.2	157.0	157.2	157.3	157.7	157.1	156.9	157.3	157.3
d, *J*(Hz)	26.3	26.5	26.2	25.2	25.3	25.2	26.2	26.3	25.2	26.2	25.2	26.2
C-5″	140.7	140.4	140.6	140.4	140.2	140.4	140.3	140.4	140.4	140.3	140.4	140.3
d, *J*(Hz)	235.8	236.9	235.8	235.8	235.2	235.8	236.9	130.1	237.3	237.0	237.3	236.2
C-6″	130.7	130.8	130.6	131.0	131.1	130.9	130.8	130.5	130.2	130.8	130.6	130.9
C-1‴	175.4	175.4	175.5	175.5	175.4	175.4	175.3	175.5	175.5	175.3	175.5	175.4
C-2‴	61.7	61.4	61.3	61.4	61.3	61.6	61.4	61.7	61.4	62.2	58.9	62.5
C-3‴	55.9	58.5	64.8	58.4	63.9	58.7	62.1	62.7	63.8	63.8	64.8	64.4
C-4‴	28.1	28.0	27.9	27.9	28.1	27.8	27.7	27.9	28.0	30.8	28.4	28.4
C-5‴											22.8	22.7
C-6‴									25.4	25.2	28.1	27.9
C-7‴	46.3	46.2	45.7	46.1	46.3	46.0	46.0	45.7	48.2	48.2	48.7	48.7
	19.5	42.7	69.1	42.6	38.7	32.7	136.6	156.4	42.7	130.8	42.6	133.8
		22.4	20.0	24.9	25.8	30.8	129.1	132.3	25.0	129.0	25.0	129.0
R		22.2		22.3	15.4	15.5	128.7	129.8	22.6	128.5	22.5	128.6
				22.2	11.5		127.1	115.3	22.4	126.9	22.3	126.9
							40.0	29.8		39.9		39.9

5.4 Hz, 1H, H-2), 3.15 (d, *J* = 5.4 Hz, 1H, H-2‴), 2.78–2.74 (m, 1H, H-3), 2.07 (t, *J* = 6.0 Hz, 1H, β-H of valine), 1.58–1.54 (m, 2H, H-4‴), 0.89 (d, *J* = 6.0 Hz, 3H, Me), 0.81 (d, *J* = 5.7 Hz, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 670.2404 for $[M+H]^+$ (calcd 670.2407 for $C_{33}H_{37}FN_3O_{11}$).

4.3.3. 4′-*O*-Demethyl-4β-*N*-[(5-FU-*N*^{1}-)-propyl L-threonyl]-4-desoxy-podophyllotoxin (12)

The yield 36%; mp: 119–121 °C; $[\alpha]_D^{23}$ −68 (*c* 0.3, acetone); IR (KBr) 3441, 3219, 3069, 2918, 2845, 1770, 1698, 1612, 1481, 1371, 1231, 1112 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.77 (br, 1H, NH), 7.35 (d, *J* = 5.1 Hz, 1H, H-6″), 6.68 (s, 1H, H-5), 6.47 (s, 1H, H-8), 6.22 (s, 2H, H-2′,6′), 5.93 (s, 2H, H-13), 5.51 (br, 1H, NH), 4.52 (d, *J* = 5.1 Hz, 1H, H-1), 4.31–4.28 (m, 3H, H-3‴, β-H of threonine), 4.17 (t, *J* = 6.0 Hz, 1H, H-11a), 4.12 (d, *J* = 3.0 Hz, 1H, H-4), 4.07 (t, *J* = 6.0 Hz, 1H, H-11b), 3.87–3.79 (m, 2H, H-7‴), 3.74 (s, 6H, 3′,5′-OMe), 3.48 (dd, *J* = 14.1, 5.1 Hz, 1H, H-2), 3.28 (d, *J* = 4.2 Hz, 1H, H-2‴), 2.90–2.86 (m, 1H, H-3), 2.08–2.06 (m, 2H, H-4‴), 1.18 (d, *J* = 6.6 Hz, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 672.2191 for $[M+H]^+$ (calcd 672.2199 for $C_{32}H_{35}FN_3O_{12}$).

4.3.4. 4′-*O*-Demethyl-4β-*N*-[(5-FU-*N*^{1}-)-propyl L-leucyl]-4-desoxy-podophyllotoxin (13)

The yield 48%; mp: 110–112 °C; $[\alpha]_D^{23}$ −65 (*c* 0.3, acetone); IR (KBr) 3436, 3223, 3072, 2957, 2844, 1773, 1715, 1699, 1660, 1482, 1368, 1231, 1115 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.87 (br, 1H, NH), 7.29 (d, *J* = 5.4 Hz, 1H, H-6″), 6.65 (s, 1H, H-5), 6.44 (s, 1H, H-8), 6.20 (s, 2H, H-2′,6′), 5.92 (s, 2H, H-13), 5.56 (br, 1H, NH), 4.48 (d, *J* = 5.1 Hz, 1H, H-1), 4.34–4.26 (m, 2H, H-3‴), 4.19 (t, *J* = 5.7 Hz, 1H, H-11a), 4.13 (t, *J* = 5.7 Hz, 1H, H-11b), 3.95 (d, *J* = 3.0 Hz, 1H, H-4), 3.83–3.74 (m, 2H, H-7‴), 3.70 (s, 6H, 3′,5′-OMe), 3.37 (dd, *J* = 13.5, 5.4 Hz, 1H, H-2), 3.29 (t, *J* = 6.0 Hz, 1H, H-2‴), 2.82–2.78 (m, 1H, H-3), 1.82–1.78 (m, 2H, β-CH_2 of leucine), 1.65–1.61 (m, 1H, CH, γ-CH_2 of leucine), 1.54–1.21 (m, 2H, H-4‴), 0.87 (d, *J* = 5.4 Hz, 3H, Me), 0.79 (d, *J* = 5.4 Hz, 3H, Me); ^{13}C NMR

(75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 684.2559 for $[M+H]^+$ (calcd 684.2563 for $C_{34}H_{39}FN_3O_{11}$).

4.3.5. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-propyl L-isoleucyl]-4-desoxy-podophyllotoxin (14)

The yield 45%; mp: 118–120 °C; $[\alpha]_D^{23}$ −81 (*c* 0.3, acetone); IR (KBr) 3439, 3229, 3072, 2963, 2840, 1773, 1714, 1700, 1663, 1613, 1481, 1369, 1231, 1114 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.28 (d, *J* = 5.1 Hz, 1H, H-6″), 6.65 (s, 1H, H-5), 6.50 (s, 1H, H-8), 6.24 (s, 2H, H-2′,6′), 5.98 (s, 1H, H-13a), 5.96 (s, 1H, H-13b), 5.38 (br, 1H, NH), 4.55 (d, *J* = 5.7 Hz, 1H, H-1), 4.33–4.30 (m, 2H, H-3‴), 4.25 (t, *J* = 6.0 Hz, 1H, H-11a), 4.20 (t, *J* = 6.0 Hz, 1H, H-11b), 3.95 (d, *J* = 4.2 Hz, 1H, H-4), 3.82 (t, *J* = 7.8 Hz, 2H, H-7‴), 3.75 (s, 6H, 3′,5′-OMe), 3.42 (dd, *J* = 13.8, 5.4 Hz, 1H, H-2), 3.29 (d, *J* = 5.4 Hz, 1H, H-2‴), 2.84–2.80 (m, 1H, H-3), 1.62–1.58 (m, 2H, H-4‴), 1.41–1.19 (m, 3H, β,γ-H of isoleucine), 0.90 (d, *J* = 3.0 Hz, 3H, Me), 0.88 (t, *J* = 3.6 Hz, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 684.2562 for $[M+H]^+$ (calcd 684.2563 for $C_{34}H_{39}O_{11}N_3F$).

4.3.6. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-propyl L-methionyl]-4-desoxy-podophyllotoxin (15)

The yield 54%; mp: 109–111 °C; $[\alpha]_D^{23}$ −68 (*c* 0.3, acetone); IR (KBr) 3438, 3229, 3069, 2915, 2840, 1773, 1715, 1698, 1660, 1612, 1481, 1369, 1231, 1113 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.31 (d, *J* = 5.4 Hz, 1H, H-6″), 6.67 (s, 1H, H-5), 6.45 (s, 1H, H-8), 6.21 (s, 2H, H-2′,6′), 5.92 (s, 2H, H-13) 4.49 (d, *J* = 5.1 Hz, 1H, H-1), 4.31 (d, *J* = 4.2 Hz, 1H, H-11a), 4.28 (d, *J* = 2.1 Hz, 1H, H-11b), 4.23–4.18 (m, 2H, H-3‴), 4.01 (d, *J* = 3.6 Hz, 1H, H-4), 3.78 (t, *J* = 6.3 Hz, 2H, H-7‴), 3.71 (s, 6H, 3′,5′-OMe), 3.46 (t, *J* = 6.0 Hz, 1H, H-2‴), 3.36 (dd, *J* = 13.8, 5.4 Hz, 1H, H-2), 2.84–2.80 (m, 1H, H-3), 2.49(t, *J* = 6.9 Hz, 2H, SCH_2), 2.05 (s, 3H, SMe), 2.11–2.08 (m, 2H, H-4‴), 1.98–1.95 (m, 2H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 702.2125 for $[M+H]^+$ (calcd 702.2127 for $C_{33}H_{37}FN_3O_{11}S$).

4.3.7. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-propyl L-phenylalanyl]-4-desoxy- podophyllotoxin (16)

The yield 54%; mp: 113–115 °C; $[\alpha]_D^{23}$ −71 (*c* 0.3, acetone); IR (KBr) 3440, 3211, 3066, 2918, 2844, 1772, 1714, 1697, 1666, 1607, 1510, 1481, 1369, 1230, 1112 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 9.58 (br, 1H, NH), 7.27–7.15 (m, 5H, Ar), 7.05 (d, *J* = 5.1 Hz, 1H, H-6″), 6.53 (s, 1H, H-5), 6.44 (s, 1H, H-8), 6.21 (s, 2H, H-2′,6′), 5.99 (s, 2H, H-13), 5.48 (br, 1H, NH), 4.44 (d, *J* = 5.1 Hz, 1H, H-1), 4.27–4.24 (m, 2H, H-3‴), 4.09 (t, *J* = 5.1 Hz, 2H, H-11), 4.01 (d, *J* = 3.3 Hz, 1H, H-4), 3.73 (s, 6H, 3′,5′-OMe), 3.67–3.48 (m, 3H, H-7‴, H-2‴), 3.15 (dd, *J* = 14.1, 5.1 Hz, 1H, H-2), 3.08–2.98 (m, 2H, β-H of phenylalanine), 2.81–2.78 (m, 1H, H-3), 1.87–1.84 (m, 2H, H-4‴); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 740.2237 for $[M+Na]^+$ (calcd 740.2226 for $C_{37}H_{36}FN_3O_{11}Na$).

4.3.8. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-propyl L-tyrosyl]-4-desoxy-podophyllotoxin (17)

The yield 27%; mp 124–126 °C; $[\alpha]_D^{23}$ −56 (*c* 0.3, acetone); IR (KBr) 3394, 3070, 2912, 2841, 1771, 1698, 1665, 1612, 1514, 1481, 1368, 1230, 1113 cm^{-1}; ^1H NMR (300 MHz, CD_3COCD_3) δ 7.78 (d, *J* = 6.6 Hz, 1H, H-6″), 7.04 (d, *J* = 8.1 Hz, 2H, Ar), 6.74 (d, *J* = 8.1 Hz, 2H, Ar), 6.71 (s, 1H, H-5), 6.43 (s, 1H, H-8), 6.28 (s, 2H, H-2′,6′), 5.95 (s, 1H, H-13a), 5.94 (s, 1H, H-13b), 4.43 (d, *J* = 5.4 Hz, 1H, H-1), 4.37–4.28 (m, 2H, H-11), 4.16–4.10 (m, 3H, H-3‴, H-4), 3.76–3.69 (m, 2H, H-7‴), 3.65 (s, 6H, 3′,5′-OMe), 3.43–3.40 (m, 1H, H-2‴), 3.24 (dd, *J* = 14.1, 5.1 Hz, 1H, H-2), 2.95–2.92 (m, 2H, β-H of tyrosine), 2.89–2.86 (m, 1H, H-3), 1.99–1.96 (m, 2H, H-4‴); ^{13}C NMR (75 MHz, CD_3COCD_3) (Table 2). HRMS (ESI): 734.2367 for $[M+H]^+$ (calcd 734.2356 for $C_{37}H_{37}FN_3O_{12}$).

4.3.9. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-butyl L-leucyl]-4-desoxy-podophyllotoxin (18)

The yield 39%; mp: 115–117 °C; $[\alpha]_D^{23}$ −62 (*c* 0.3, acetone); IR (KBr) 3438, 3222, 2957, 2843, 1772, 1711, 1698, 1613, 1511, 1482, 1367, 1231, 1114 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 9.58 (br, 1H, NH), 7.27 (d, *J* = 6.0 Hz, 1H, H-6″), 6.66 (s, 1H, H-5), 6.47 (s, 1H, H-8), 6.24 (s, 2H, H-2′,6′), 5.95 (s, 2H, H-13), 5.45 (br, 1H, NH), 4.51(d, *J* = 5.4 Hz, 1H, H-1), 4.37 (t, *J* = 6.3 Hz, 2H, H-11), 4.29–4.14 (m, 2H, H-3‴), 3.95 (d, *J* = 3.6 Hz, 1H, H-4), 3.80 (t, *J* = 7.2 Hz, 2H, H-7‴), 3.75 (s, 6H, 3′,5′-OMe), 3.39 (dd, *J* = 13.5, 5.1 Hz, 1H, H-2), 3.36–3.28 (m, 1H, H-2‴), 2.82–2.80 (m, 1H, H-3), 1.76–1.74 (m, 2H, β-H of leucine), 1.65–1.61 (m, 1H, γ-H of leucine), 1.57–1.55 (m, 4H, H-4‴,6‴), 0.90 (d, *J* = 5.4 Hz, 3H, Me), 0.83 (d, *J* = 6.0 Hz, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 698.2732 for $[M+H]^+$ (calcd 698.2720 for $C_{35}H_{41}FN_3O_{11}$).

4.3.10. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-butyl L-phenylalanyl]-4-desoxy- podophyllotoxin (19)

The yield 43%; mp: 111–113 °C; $[\alpha]_D^{23}$ −68 (*c* 0.3, acetone); IR (KBr) 3442, 3184, 3063, 2920, 1773, 1715, 1698, 1609, 1506, 1481, 1365, 1230, 1112 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.26–7.12 (m, 6H, H-6″, Ar), 6.46 (s, 1H, H-5), 6.41 (s, 1H, H-8), 6.19 (s, 2H, H-2′,6′), 5.91 (s, 2H, H-13), 4.89 (br, 1H, NH), 4.41 (d, *J* = 5.4 Hz, 1H), 4.25–4.22 (m, 2H, H-11), 4.09–4.05 (m, 2H, H-3‴), 3.97 (d, *J* = 3.3 Hz, 1H, H-4), 3.71 (s, 6H, 3′,5′-OMe), 3.63–3.60 (m, 2H, H-7‴), 3.49 (t, *J* = 6.6 Hz, 1H, H-2‴), 3.13 (dd, *J* = 13.8, 5.7 Hz, 1H, H-2), 2.97 (d, *J* = 7.2 Hz, 1H, β-H of phenylalanine), 2.77–2.74 (m, 1H, H-3), 1.57–1.55 (m, 4H, H-4‴,6‴); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 732.2555 for $[M+H]^+$ (calcd 732.2563 for $C_{38}H_{39}FN_3O_{11}$).

4.3.11. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-pentyl L-leucyl]-4-desoxy-podophyllotoxin (20)

The yield 35%; mp: 106–108 °C; $[\alpha]_D^{23}$ −65 (*c* 0.3, acetone); IR (KBr) 3429, 3214, 3070, 2954, 2870, 1773, 1715, 1697, 1613, 1481, 1365, 1230, 1114 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 9.56 (br, 1H, NH), 7.36 (d, *J* = 5.1 Hz, 1H, H-6″), 6.70 (s, 1H, H-5), 6.49 (s, 1H, H-8), 6.26 (s, 2H, H-2′,6′), 5.98 (s, 2H, H-11), 5.52 (br, 1H, NH), 4.54 (d, *J* = 6.0 Hz, 1H, H-1), 4.37 (t, *J* = 6.3 Hz, 2H, H-3‴), 4.21 (t, *J* = 5.4 Hz, 1H, H-11a), 4.11 (t, *J* = 5.4 Hz, 1H, H-11b), 3.97 (d, *J* = 3.3 Hz, 1H, H-4), 3.76 (s, 6H, 3′,5′-OMe), 3.73 (t, *J* = 6.6 Hz, 2H, H-7‴), 3.42 (dd, *J* = 14.1, 5.4 Hz, 1H, H-2), 3.29 (t, *J* = 6.0 Hz, 1H, H-2‴), 2.84–2.81 (m, 1H, H-3), 1.75–1.72 (m, 2H, β-H of leucine), 1.68–1.65 (m, 1H, γ-H of leucine), 1.65–1.62 (m, 4H, H-4‴,6‴), 1.25–1.23 (m, 2H, H-5‴), 0.92 (d, *J* = 6.0 Hz, 3H, Me), 0.87 (d, *J* = 6.0 Hz, 3H, Me); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 712.2883 for $[M+H]^+$ (calcd 712.2876 for $C_{36}H_{43}FN_3O_{11}$).

4.3.12. 4′-*O*-Demethyl-4β-*N*-[(5-FU-N^1-)-pentyl L-phenylalanyl]-4-desoxy- podophyllotoxin (21)

The yield 24%; mp: 125–127 °C; $[\alpha]_D^{23}$ −61 (*c* 0.3, acetone); IR (KBr) 3443, 3066, 2938, 2837, 1774, 1714, 1693, 1613, 1505, 1428, 1366, 1231, 1114 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 9.64 (br, 1H, NH), 7.28–7.12 (m, 6H, H-6″, Ar), 6.47 (s, 1H), 6.42 (s, 1H), 6.20 (s, 2H, H-2′,6′), 5.93 (s, 2H, H-13), 5.49 (br s, 1H, NH), 4.43 (d, *J* = 5.4 Hz, 1H, H-1), 4.27(d, *J* = 9.0 Hz, 2H, H-11), 4.08–4.05 (m, 2H, H-3‴), 3.97 (d, *J* = 2.7 Hz, 1H, H-4), 3.72 (s, 6H, 3′,5′-OMe), 3.64 (t, *J* = 7.2 Hz, 2H, H-7‴), 3.49 (t, *J* = 6.0 Hz, 1H, H-2‴), 3.14 (dd, *J* = 14.1, 5.7 Hz, 1H, H-2), 2.98 (d, *J* = 6.0 Hz, 2H, β-H of phenylalanine), 2.78–2.75 (m, 1H, H-3), 1.63–1.60 (m, 4H, H-4‴,6‴), 1.27–1.25 (m, 2H, H-5‴); ^{13}C NMR (75 MHz, $CDCl_3$) (Table 2). HRMS (ESI): 746.2705 for $[M+H]^+$ (calcd 746.2720 for $C_{39}H_{41}FN_3O_{11}$).

4.4. Octanol–water partition coefficients

The partition coefficient *P* was determined according to the standard method[25] and calculated with following equation: *P* = (compound in 1-octanol)/(compound in water). The reported *P* values are an average of three measurements.

4.5. Biology

4.5.1. Cytotoxicity assays

Cytotoxicity assays are performed on human premyelocytic leukemia (HL-60), human erythromyeloblastoid leukemia (K562), human gastric cancer (AGS) and human lung carcinoma (A-549). Cells (6000–10,000) in 100 μL culture medium per well were seeded into 96-well microtest plates (Falcon, CA). Cells were treated in triplicate with gradient concentration of test compounds and incubated at 37 °C for 48 h (or 72 h). For HL-60 and K562 cell lines, the microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; Sigma, St. Louis, MO] assay was performed to measure the cytotoxic effects. The growth inhibitory effect on AGS and A549 solid tumour cell lines was measured by the Sulforhodamine B (SRB; Sigma, St. Louis, MO) assay. The drug concentration required for 50% growth inhibition (IC_{50}) of tumour cells was determined from the dose–response curve.

4.5.2. Circular dichroism (CD) studies of CT DNA

CT DNA was dissolved and annealed in 10 mM PBS (pH 7.2). To CT DNA in PBS (5 mL 50 μg/mL) was added compound **21** in PBS (0, 1, 2, 5 mL 100 μg/mL, respectively), and diluted to 10 mL. Same concentration sample of **21** in PBS was also prepared. The above mixture was incubated for 24 h in the dark at 37 °C. Circular dichroism (CD) spectra were measured using a JASCO-810 spectrometer at 20 °C in quartz cells of 1 cm path length. Spectra of CT DNA in PBS were recorded in the range 200–320 nm. The appropriate concentration samples of compound **21** in PBS were also determined as blank.

4.5.3. Stability studies for compound 21[26]

For drug metabolism experiments, to 4.0 mL preincubated human plasma, mouse plasma and pH 7.4 phosphate buffer, respectively, was added test compound **21** (100 μg/mL, 1.0 mL) in acetonitrile. The mixture was incubated at 37 °C, and 100 μL aliquots were taken at 0.5, 1, 2, 4, 6, 8, 12 and 24 h. To precipitate plasma protein, 400 μL acetonitrile (−20 °C) was added, vortexed for 20 s and centrifuged at 10,000 rpm for 5 min. Supernatant was transferred to a glass vial and stored at −20 °C immediately until HPLC analysis. HPLC analysis: 20 μL of solution obtained above was injected onto a Kromasil 100 A C18 column (350 mm × 4.6 mm, 5 μm), chromatographed with acetonitrile–water as mobile phase (CH_3CN/H_2O = 45:55) and flow rate is 1.0 mL/min. Compound **21** was detected with SPD-M10AVP UV–vis detector at 278 nm and remaining time is 10.7 min.

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