Biological Chemistry & Chemical Biology

Synthesis and Antiproliferative Activity of 4β-*O*-Substituted, 4β-*N*-Substituted Deoxypodophyllotoxin Derivatives, and 4β-*OH*- 4′-*O*-Substituted Podophyllotoxin

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Podophyllotoxin (PPT) and its derivatives possess various biological activities and particularly against numerous cancers. The high toxicity and side effects of this therapeutic class are behind the restricted deployment in clinical applications. Here, we have identified and reported the synthesis of 4β-O-substituted 4′-O-demethylpodphyllotoxin derivatives, 4β-N-substituted 4′-O-demethyl-4- deoxypodophyllotoxin derivatives and 4β-OH- 4′-O-substituted podophyllotoxin derivative (series a, e and p) respectively. These derivatives are either esters or amides of various heterocyclic moieties which include imidazole, thiazole, pyrazole, and indole. Their synthesis was carried out in one step reaction and obtained in good yield. We tested all prepared compounds against T47D, MDA231, Caco-2, and MCF-7 cancer cell lines. They have been shown to possess significant biological activity, where series "a" has been demonstrated to be the most potent one. Compound **3a** exhibits potential activity in the micromolar range scoring 11 μM against Caco-2 and 18 μM against MDA231. Consequently, all synthesized compounds have been evaluated for their safety profile and tested against PCS201012 which are normal skin fibroblast cell lines showing noticeable safety. The biological results demonstrate that the presence of an aromatic heterocycle ring in position 4 and 4′ lead to derivatives less toxic compared to podophyllotoxin and epipodophyllotoxin. Also, they are less potent. However, the results obtained will have, in future work, an important impact on the development of more potent and less toxic leads.

Introduction

Podophyllotoxin (PPT), a natural lignin, and its 4β epimers: compound **1** (4′-O-substituted 4′-O-demethylpodphyllotoxin) and compound **2** (4′-N-substituted 4′-O-demethyl-4-deoxypodophyllotoxin) (figure 1) possess various biological activities including antineoplastic and antiviral.[1,2] The antineoplastic activity of PPT is based on the inhibition of tubulin assembly into microtubules.[3,4] However, Its undesirable effects; especially gastrointestinal toxicity; hampered its clinical use.[5] Whereas, epipodophyllotoxin derivatives, which are semi-synthetic epimers of PPT (4β epimers) possess a potent inhibitory effect on the DNA topoisomerase II.[6] They are known to block the catalytic activity of DNA topo-II by stabilizing an enzyme–DNA-complex in which the DNA is cleaved and covalently linked to an enzyme.[7]

Currently, all clinically used agents related to PPT belong to the epipodophyllotoxin epimer, these include etoposide (Etopophos®), teniposide (Vumon®), and the prodrug etopophos (figure 2).[2] These derivatives are employed either as a single therapeutic agent or in a combination with other chemotherapeutics for the treatment of small-cell lung cancer, acute leukemia, lymphoma, testicular carcinoma, and Kaposi's Sarcoma.[8]

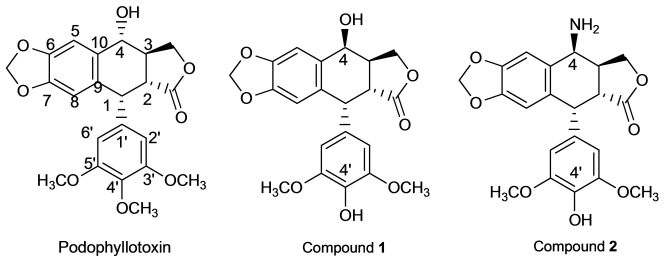

Figure 1. Structures of Podophyllotoxin (PPT) and compounds **1** and **2**.

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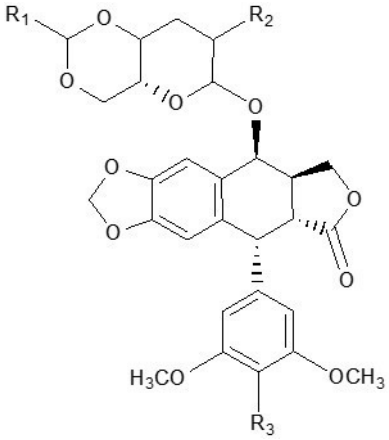


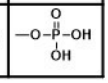
	R_1	R_2	R_3
Etoposide	$-CH_3$	—OH	—OH
Teniposide		—OH	—OH
Etopophos	$-CH_3$	—OH	

Figure 2. Structures of epipodophyllotoxin derivatives in clinical use.

A well-documented structure-activity relationship of epipodophyllotoxin derivatives and comparative molecular field analysis ('CoMFA') models for topoisomerase II inhibition clearly showed that carbon 4 (figure 1) is the most active site for any structural modification, and is considered a key character in the DNA topo-II inhibition.[6,9,10] It is well-documented that the 4′-methoxy group is important for the anti-tubulin activity, whilst the 4′-demethoxy group (4′-OH) is beneficial to the topoisomerase inhibitory activity.[11] Any modification in position 4′ is usually intended to produce a prodrug or a hybrid drug, however, published research has shown that substitution at the former position can produce agents with good antiproliferative properties.[12]

Recently, derivatives of 4β-*N*-substituted 4′-*O*-demethyl-4-deoxypodophyllotoxin (compound **2**) (figure 1) have exhibited considerable cytotoxic properties and improved binding abilities to DNA topo-II compared with etoposide.[2,13] In the 4β-*N*-substituted 4′-*O*-demethyl-4-deoxypodophyllotoxin class, various heterocyclic nuclei at C4 were tested for their cytotoxic activity as in the case of substituted furan ring. These derivatives have been tested against HeLa, K562 and K562/A02 cancer cell lines and results showed that their cytotoxic activity was comparable to etoposide or even better.[14] Furthermore, triazole derivatives were designed and tested against previous cancer cell lines showing biological activity superior to etoposide.[7] Similarly, a new series of 4β-(thiazol-2-yl)amino-4′-*O*-demethyl-4-deoxypodophyllotoxins were also synthesized and tested against A549, HepG2, HeLa, and LOVO cancer cell lines showing the ability to induce DNA double-strand breaks *via* the p73/ATM pathway as well as the H2AX phosphorylation in A549 cells.[6]

In the present work, we are reporting the synthesis of 4β-*O*-substituted 4′-*O*-demethylpodphyllotoxin (the "e series") and 4β-*N*-substituted 4′-*O*-demethyl-4- deoxypodophyllotoxin derivatives (the "a series") linked through an ester and an amide functional groups to the heterocycles imidazole, thiazole, oxazole, pyrazole, and indole respectively using a direct one-step coupling reaction of compounds **1** or **2** with the corresponding heterocyclic acyl chloride.

The third series of compounds (the "p series") synthesized through the reaction of the acyl chloride of the former heterocycles with the phenolic OH in the position 4′ producing the 4β-*O*H- 4′-*O*-substituted podophyllotoxin which was also investigated for its biological activity showing potential for development. This research aims to evaluate the biological activity of the synthesized compounds using MTT test and flow cytometry and to evaluate the effects of the substitution in position 4′ and its impact on the biological activity.

Results and Discussion

Chemistry

All desired compounds were synthesized by reacting the acyl chlorides of different heterocycles with epipodophyllotoxins **1** and **2** providing the corresponding esters and amides in a one-pot reaction as depicted in schemes 1 and 2. This methodology was frequently used by our team for the synthesis of various products and represents a direct and less time-consuming approach compared to other synthetic procedures.[15,16] It is observed that due to the presence of more than one reactive hydroxyl in compound **1**, which is the alcoholic OH in position 4 and the phenolic OH in position 4′, a mixture of products was obtained in each reaction (detected by TLC) therefore the most intense spot was isolated from each reaction using plate chromatography technique. All synthesized compounds were later separated using chromatographic methods and identified through ^{1}H NMR, ^{13}C NMR, HRMS. All data regarding the identification, purification, and determination of these compounds are reported in the experimental part located in the supplementary material.

Biology

MTT assay

Our aim in the present work was to investigate the antineoplastic potential of the prepared compounds, therefore an MTT

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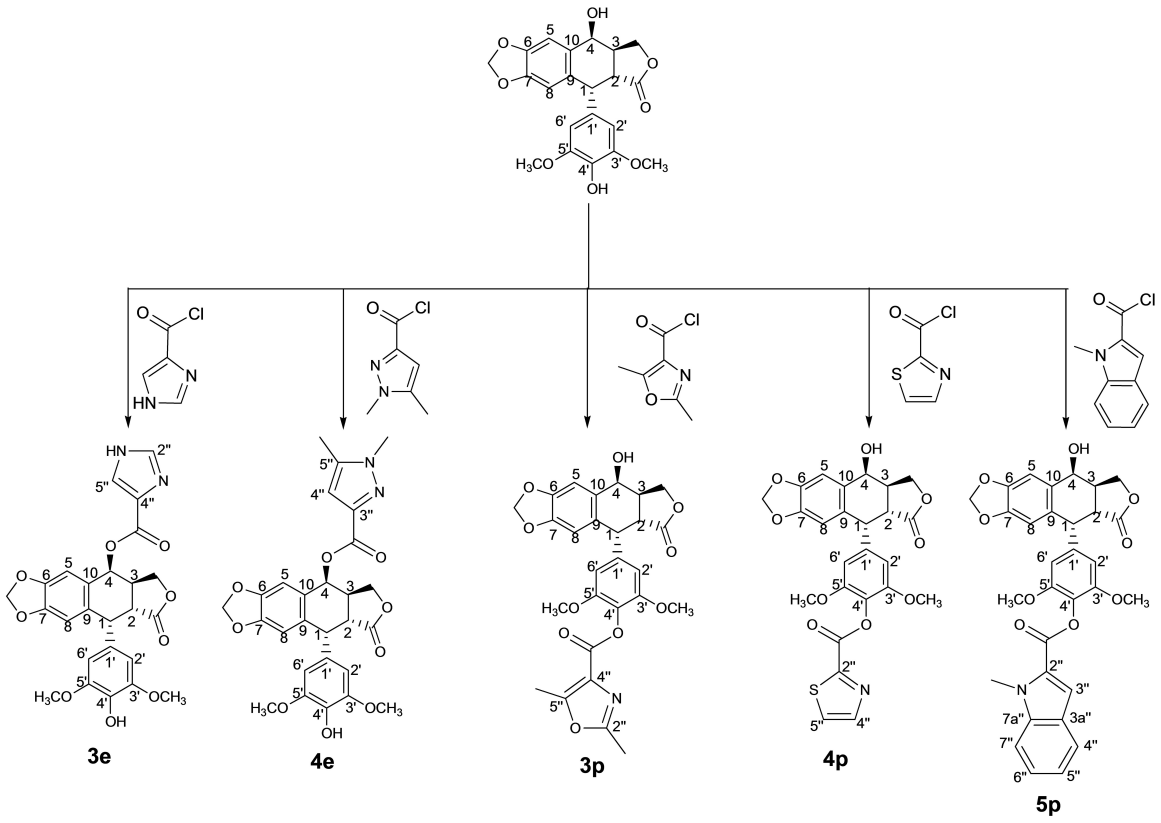
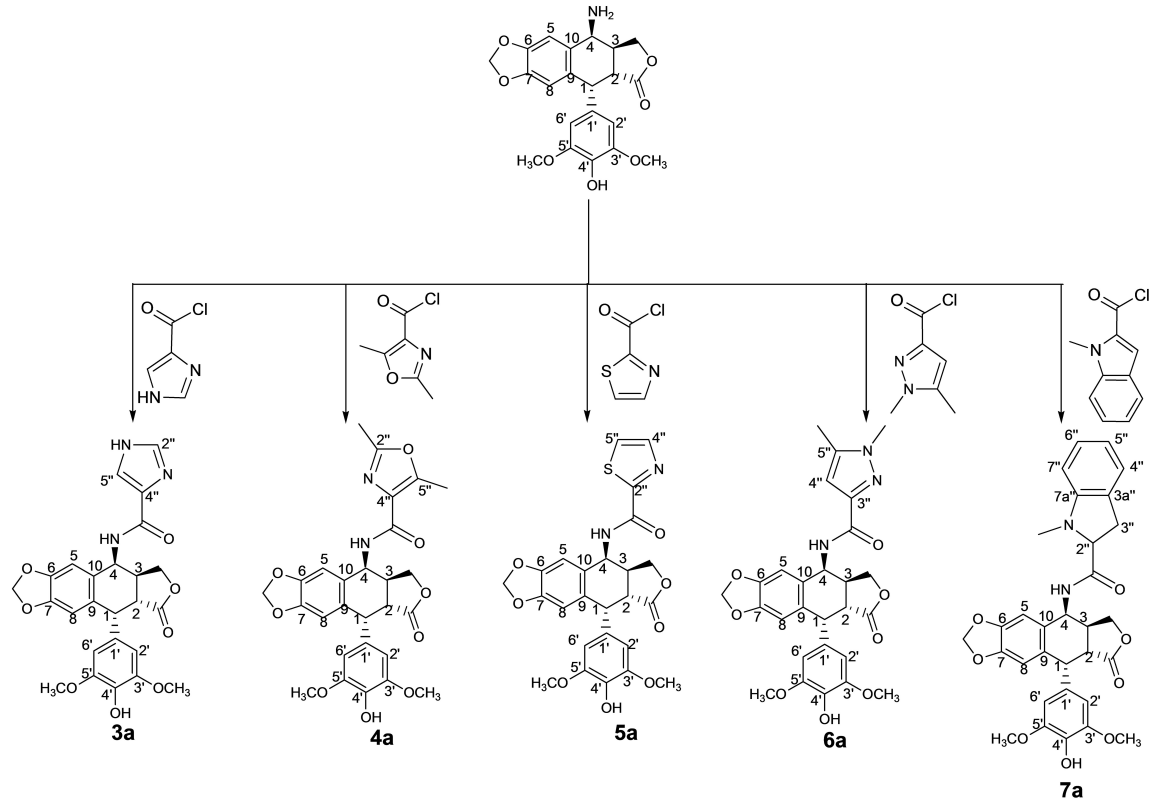


Scheme 1. Synthesis of 4β-O-substituted 4′-O-demethylpodphyllotoxin **3 e**, **4 e**, and 4β-OH- 4′-O- substituted demethylpodophyllotoxin **3 p**–**5 p**.



Scheme 2. Synthesis of 4β-N-substituted 4′-O-demethyl-4- deoxypodophyllotoxin derivatives **3 a**–**7 a**.

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assay was performed to evaluate their antiproliferative properties.

As described in the experimental section (supplementary material), the MTT test was performed against four cancer cell lines specifically MCF-7, Caco-2, MDA231, T47D,[17,18] and one normal cell line, namely skin Fibroblasts (PCS201012).[19,20] which was selected as a reference of normal cells to evaluate the safety of our products as reported in table 1.

Results of MTT assay showed good tolerability of the synthesized compounds in normal cells. The improved tolerability was accompanied by a reduction in the cytotoxic effect of the various derivatives compared to PPT and reference compounds **1** and **2**.

The most potent compounds in the various series were derivatives of the "a series" (4β-amino-podophyllotoxin derivatives) and the most potent compound in this series was compound **3 a** in all examined cell lines which is a 4β-amino-podophyllotoxin containing imidazole ring as a substituent in position 4. Compound **3 a** shows also a reasonable safety margin displaying a significantly reduced toxicity to the normal skin fibroblast cells. The potency of compound **3 a** is twice the potency of the nearest derivative of the series in all cell lines. We also observe that compound **7 a** which is the second-highest potent derivative belongs also to "a series" containing the heterocycle N-methylindole in position 4. We can assume that the increased potency of compound **3 a** is related to the increased water solubility of the imidazole substituent in comparison with other derivatives in the same series. Alternatively, the relatively high potency of compound **7 a** may be due to the increased bulkiness of the methylindole substituent which is considered a major factor in the topoisomerase affinity.

In general, results of the antiproliferative activity for 4β-O-podophyllotoxin derivatives "e series" indicated less potency than 4β-amino derivatives in most tested cancer cell lines but once again the most potent compound in the "e series" was the one containing an imidazole moiety.

In the " p series", the most potent compound was **5 p** which had comparable potency to the compound **7 a** which possesses the same heterocycle (methylindole) linked in position 4 through an amide linkage. This type of dualism in the activity was also observed between **5a** with **4 p** and **4 a** with **3 p**. All these pairs possess the same heterocycle linked in different positions and types of linkage, i.e. as esters or amides in position 4 or as ester in position 4′ (schemes 1 and 2). We observe that the biological activity of the "p series" is comparable to the other series in contrast with the literature indicating that substitution in position 4′ would negatively affect the biological activity.

Flow cytometry

To determine if the reported anti-proliferative effect of the examined series is mediated by an apoptotic pathway, flow cytometry technology was employed on T47d cells treated with 50 μM of compound **3 a**.[21] The dot plots (Figure 3) demonstrate the percentages of apoptosis and necrosis of each cell population under the different conditions of treatment. Treating cells with compound **3 a** significantly increased the percentage of cells undergoing apoptosis compared to the negative control. Further investigation should shed light on the possible molecular mechanisms through which apoptosis pathways are triggered upon treatment with compound **3 a**. Details of the methodology are reported in the experimental section found in the supplementary material

Conclusion

Podophyllotoxin remains a valuable lead in the design and synthesis of highly potent antineoplastic agents. The epipodophyllotoxin derivatives, etoposide, and teniposide are in clinical use against numerous cancers. Tremendous and extensive research has been conducted during the past years aimed at the development of new derivatives with higher potency, broader spectrum, and mainly safer and more tolerable agents. For the same purpose, in this research, we have identified and prepared three series of epipodophyllotoxin derivatives, specifically the 4β-O-substituted 4′-O-demethylpodphyllotoxin (the

Table 1. The 50% inhibitory concentration (IC_{50}) against different human cancer cells and skin fibroblasts normal cells (PCS-201012). Values are presented as mean (μM) ± SD of three independent experiments.

Compound	MCF-7	Caco-2	MDA231	T47D	PCS-201012
3 e	52 ± 2	44 ± 5	49 ± 2	53 ± 4	> 100
4 e	78 ± 3	65 ± 5	88 ± 6	79 ± 5	> 100
3 p	82 ± 3	77 ± 6	72 ± 3	79 ± 5	> 100
4 p	55 ± 1	49 ± 2	46 ± 2	53 ± 3	> 100
5 p	48 ± 1	50 ± 1	39 ± 3	47 ± 2	> 100
3 a	25 ± 2	11 ± 1	18 ± 3	27 ± 1	> 100
4 a	75 ± 5	65 ± 4	69 ± 4	71 ± 3	> 100
5 a	52 ± 3	42 ± 2	56 ± 5	51 ± 4	> 100
6 a	69 ± 4	53 ± 3	71 ± 3	66 ± 2	> 100
7 a	49 ± 4	34 ± 4	33 ± 4	48 ± 2	> 100
PPT[a]	0.15 ± 0.01	0.11 ± 0.03	0.14 ± 0.05	0.14 ± 0.03	2.3 ± 0.08
1	1.5 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	2.8 ± 0.1
2	2.1 ± 0.2	1.8 ± 0.1	2.0 ± 0.3	1.7 ± 0.3	3.1 ± 0.2

[a]: Podophyllotoxin.

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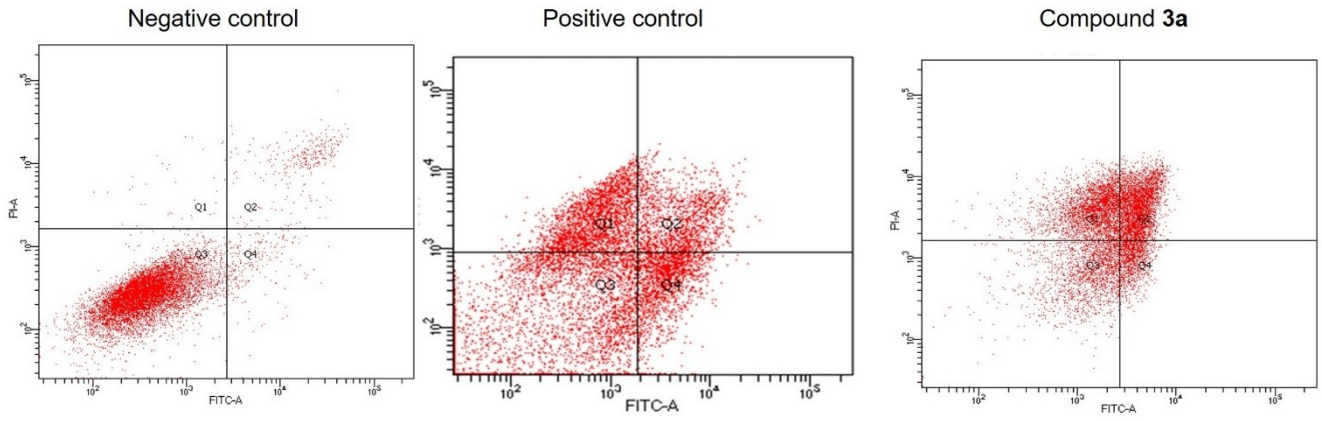

Figure 3. Dot plots of flow cytometry on T47d cell line. T47D breast cancer cells untreated as a negative control or treated with Doxorubicin (5 μM) as a positive control, or compound **3 a** (50 μM) for 72 h then stained with annexin V-FITC/ PI and analyzed by flow cytometry: where Q3 showed viable cells, Q1 necrotic cells, Q2 late apoptotic, and Q4 early apoptotic cells in T47D.

"e series") and 4β-N-substituted 4'-O-demethyl-4- deoxypodophyllotoxin derivatives (the "a series") and the 4β-O-4'-O-demethyl substituted podophyllotoxin series (the "p series"). The potential of the synthesized compounds as anticancer agents was evaluated against MCF-7, Caco-2, MDA231, and T47D cancer cell lines. We have determined that the derivatives containing an amide linkage in position 4 (the "a series") were the most potent, in which compound **3 a** manifested the highest activity among all derivatives. This result might be attributed to the amide linkage which increases the affinity toward topoisomerase II as reported by earlier literature, and to the presence of the imidazole substituent which provides improved water solubility. We have also determined that the biological activity of the "p series" is comparable to the other series in contrast with the literature indicating that substitution in position 4' would negatively affect the biological activity. Flow cytometry test performed for compound **3 a** showed that apoptosis mediated pathways appear to be involved in the underlying mechanism of the observed anticancer activity.

Our findings demonstrate that these compounds will guide us and other researchers to develop new epipodophyllotoxin derivatives in possession of more desirable biological activities compared to those already in clinical use.

Supporting Information Summary

The following are available as supporting information: experimental section including Instrumentation, General Procedure for the synthesis, ^{1}H, ^{13}C NMR, HRMS, yield and separation methods for all prepared compounds, Cells and cell culture conditions, Cell Viability Assay (MTT), Flow cytometry methodology.

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Conflict of Interest

The authors declare no conflict of interest.

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