Bioorganic & Medicinal Chemistry Letters 22 (2012) 2590–2593

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and comparative evaluation of 4-oxa- and 4-aza-podophyllotoxins as antiproliferative microtubule destabilizing agents

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ARTICLE INFO

Article history:
Received 9 December 2011
Revised 26 January 2012
Accepted 30 January 2012
Available online 9 February 2012

Keywords:
Oxa-podophyllotoxins
Aza-podophyllotoxins
Microtubule destabilizing agents
Sea urchin embryo
Cytotoxicity

ABSTRACT

A series of novel 4-oxa-podophyllotoxin derivatives **7** featuring the intact lactone ring D and various substituents in rings B and E has been synthesized and evaluated in a phenotypic sea urchin embryo assay along with the representative 4-aza-analogs 5 for their antimitotic and microtubule destabilizing activity. The most active compounds exhibited myristicin-derived or a 3′,5′-dimethoxy substitution pattern in the ring E and a 6-methoxy moiety replacing the methylenedioxy ring A. Compounds **5xb**, **5xe**, **5yb**, **7xa**, **7xb**, and **7xc** showed potent antiproliferative effects in the NCI60 cytotoxicity screen. Notably, growth of the multi-drug resistant NCI/ADR-RES cells was more affected by these agents than the parent OVCAR-8 cell line. Although generally 4-oxa-podophyllotoxins were less potent than the respective 4-aza-derivatives in these assays, stability of the former series towards oxidation may prove to be of interest for the development of anticancer agents with in vivo activity.

Podophyllotoxin (PT) (Fig. 1) is a natural aryltetralin lignan found in plants of genus *Podophyllum* as well as in other species. It has been described to be a strong microtubule destabilizing agent that binds to the colchicine site of tubulin.[1–4] Furthermore, PT has been shown to be a bi-functional ligand interacting with multiple tubulin sites similar to colchicine.[5,6] Several semisynthetic PT derivatives, including etoposide, teniposide, and etopophos (Fig. 1), are antitumor agents currently in clinical use for the treatment of various malignancies.[3,7,8]

There are four contiguous stereocenters in PT (Fig. 1). Epimerization of the C_1 center substituted with a thrimethoxyphenyl group in the parent molecule proved to be an issue in the structure–activity studies of PT derivatives.[2] Several teams described heterocyclic analogs of PT that do not undergo epimerization and modified in the ring C. For example, 4-aza-PT[9–19] and 4-oxa-PT[13,20,21] derivatives feature a single chiral center at C_1 (Fig. 1) and are easily accessible. They exhibited a broad spectrum of biological activities, including insecticidal potential,[13] cancer cell growth inhibition,[9,11,12,14–18,20,21] cell cycle arrest in the G2/M phase,[16,18,20] inhibition of tubulin polymerization,[14,16,17,20] cellular microtubule disassembly,[17,18] caspase-3 dependent apoptosis,[11,16–18] and vascular disruption effect.[14] Among 4-aza-PTs made from the easily available plant polyalkoxybenzenes myristicin (**1b**) and apiol (**1d**) (Scheme 1),[18,22] the most potent analogs contained the myristicin-derived ring E (**II**; Fig. 1).[18] Notably, the methylenedioxy ring A was not a prerequisite for their activity, since 6-methoxy derivatives **5xb** and **5xe** displayed strong antiproliferative effect. These molecules were more potent in the sea urchin embryo assay than the parent PT.[18] Compounds **5xa** and **5xe** also displayed pronounced larvicidal effects.[13] Notably, our previous studies showed that while 4-aza-PTs **I**, **II**, and **5** were stable as solids during storage at room temperature, they underwent rapid oxidation to furnish inactive quinolines **4** in DMSO or ethanol.[18] Therefore in this paper we focused our attention on respective 4-oxa-analogs **7** that are less likely to be unstable under these conditions.

It was reported previously that 4-oxa-PT inhibited cancer cell growth at concentrations of 0.1–1 μM,[21] whereas 4-oxa-2-aza-PT was cytotoxic with IC_{50} = 0.077 μM.[21,23] These compounds were less active than the parent PT (IC_{50} = 10–40 nM (Table S1, Supplementary data)). It is worth emphasizing that a replacement of

Abbreviations: PT, podophyllotoxin.

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doi:10.1016/j.bmcl.2012.01.128

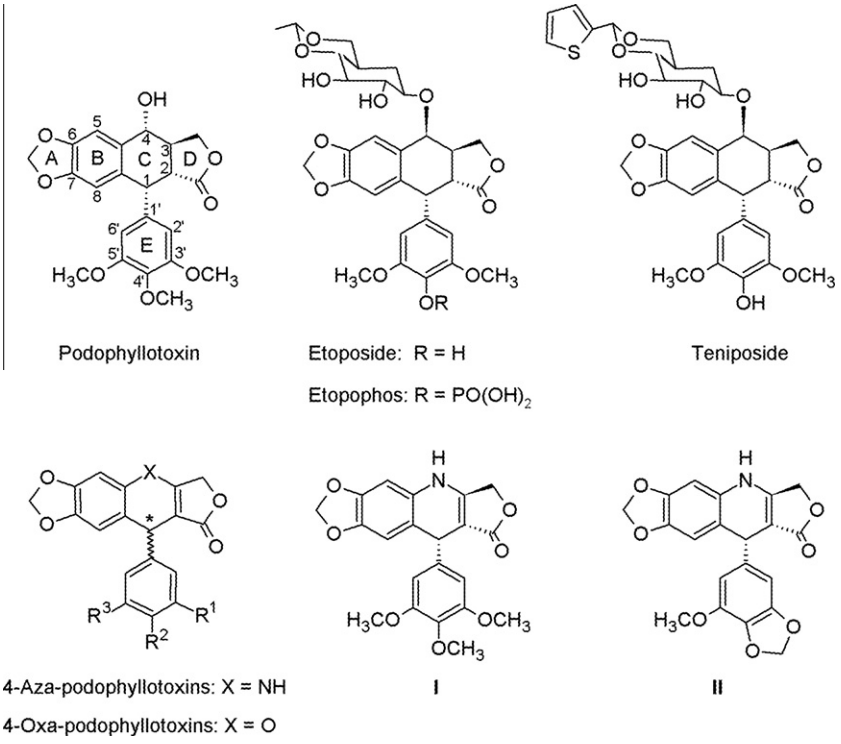


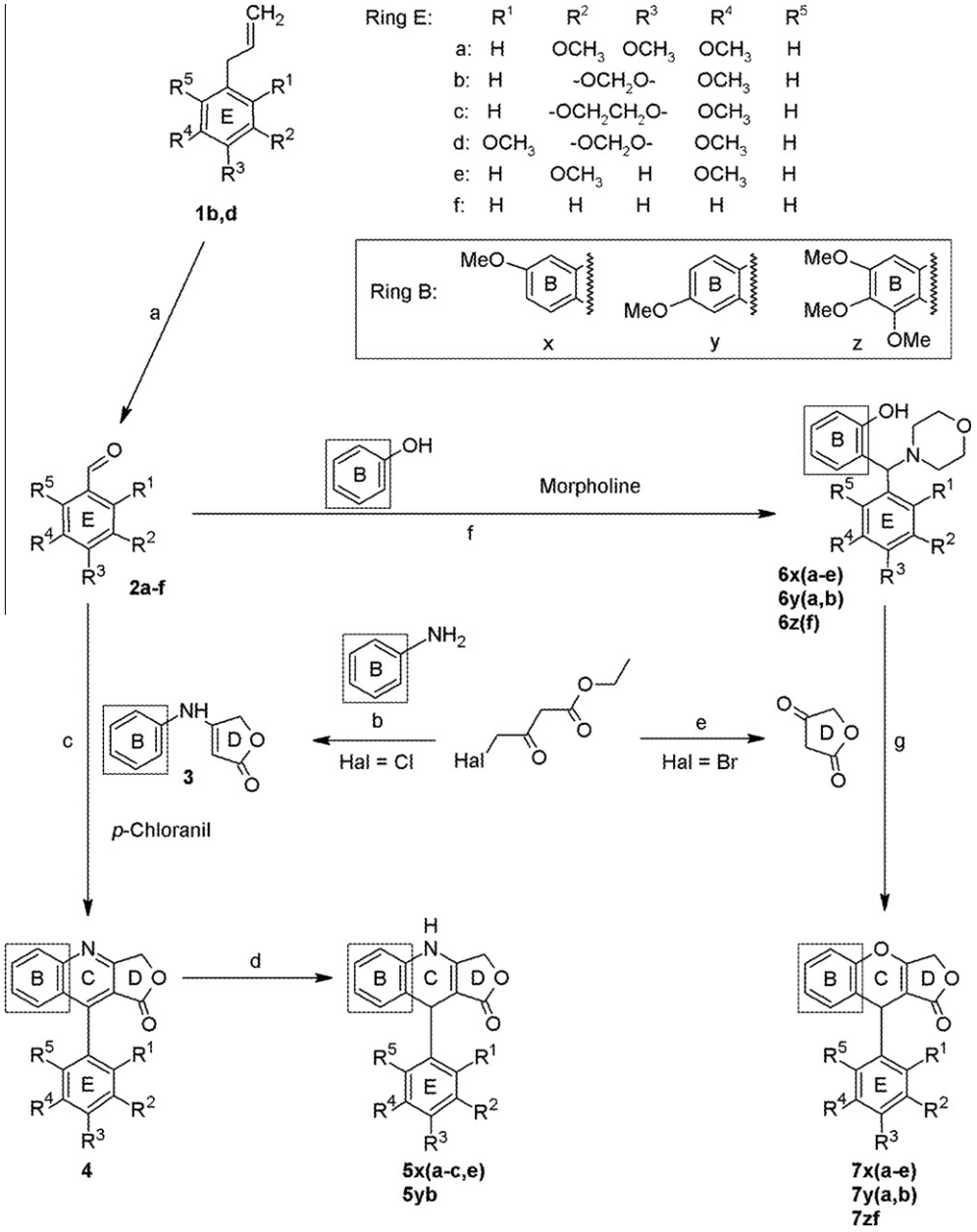
Figure 1. Structures of podophyllotoxin and its analogs.

the dihydropyridine ring C in aza-PTs with pyran significantly decreased larvicidal activity.[13] On the other hand, methylenedioxybenzopyran analogs of PT substituted at positions C_2 and C_3 versus the lactone ring D inhibited tubulin polymerization, competitively blocked colchicine binding to tubulin, and displayed cytotoxicity against murine leukemia cells in a submicromolar concentration range.[20] In this respect, it should be emphasized that the lactone ring D is essential for binding to tubulin,[6] and its modifications led to decreased cytotoxicity of the resulting derivatives.[3,24,25] Therefore, the aim of this study was to prepare mainly 4-oxa- and several 4-aza-PT derivatives with the intact lactone ring D, while varying substituents in the rings B and E, and to compare the resultant series in a panel of biological assays.

A two-steps synthesis of aromatic aldehydes (**2b**, **2d**) from natural allylbenzenes (**1b**, **1d**) and involving alkaline double bond migration followed by ozonolysis was reported previously.[26] Plant-derived (**2b**, **2d**) and commercial (**2a**, **2c**, **2e**, **2f**) aldehydes served as key intermediates in our synthetic approach as described in Scheme 1.[22] In an effort to synthesize 4-oxa-PT derivatives **7** we were unable to reproduce published synthetic strategy towards key intermediates **6** using MeOH as a solvent.[13] Gratifyingly, these targeted Mannich bases were successfully accessed via a condensation reaction of aromatic aldehydes (**2a–f**), phenols, and morpholine in absolute toluene under N_2 in a reactor equipped with a moisture trap filled with an anhydrous Na_2SO_4 and a condenser. In the next step, the molecules **7** (3.2–35% yield) were subsequently obtained by reacting **6** and tetronic acid.[13,21,27] An alternative synthetic route to **7xe** was reported earlier.[13] 4-Aza-PTs (**5**) were synthesized as reported previously[18] by cyclizing 4-Cl-acetoacetic acid with amines to afford anilinolactones (**3**).[28] These intermediates were further reacted with aldehydes (**2a–e**) in the presence of *p*-chloranil as an oxidizing agent to furnish quinoline derivatives **4**. Quinolines **4** were subsequently hydrogenated with $NaBH_3CN$ in a glacial acetic acid to afford the targeted 1,4-dihydropyridines **5** (43–73% yield). Further experimental details describing synthetic protocols and analytical data are summarized in the Supplementary data.

All molecules were evaluated in a phenotypic sea urchin embryo assay[29] in order to assess their antimitotic and microtubule destabilizing activities using PT as a benchmark reference compound (Table 1). The assay includes (i) fertilized egg test for antimitotic activity displayed by cleavage alteration/arrest, and (ii) behavioral monitoring of a free-swimming blastulae treated immediately after hatching. We have shown earlier that lack of forward movement, settlement to the bottom of the culture vessel, and rapid spinning of embryos around the animal-vegetal axis suggests a microtubule destabilizing activity caused by a test article.[29,30] In our hands, data generated from the sea urchin embryo assay, purified tubulin polymerization screen, and microtubule disruption in human cancer cells correlated well.[18,31,32] The activity was quantified as an effective threshold concentration (EC) resulting in cleavage alteration, cleavage arrest, or embryo spinning. At these concentrations, all tested molecules caused 100% effect, whereas at twofold lower concentrations, the compounds failed to produce any developmental changes. Further details on the sea urchin embryo assay are summarized in the Supplementary data. The cytotoxicity of compounds **5xb** and **7xb** was further assessed in Jurkat leukemia T-cells and the NCI60 human tumor cells.[33]

As shown in Table 1, all tested molecules except for **7zf** caused a noticeable cleavage alteration, arrest, and embryo spinning, suggesting their direct microtubule destabilizing activity. 4-Aza-PTs (**II**, **5xb**, **5xc**, **5xe**, **5yb**) and 4-oxa-PTs (**7xa**, **7xb**, **7xc**, **7xe**) displayed higher antimitotic activity than the parent PT. 4-Oxa-derivatives were somewhat less potent than the respective aza-analogs. Myristicin derivatives of both 4-aza and 4-oxa PTs were generally more active than the corresponding trimethoxyphenyl analogs (compare: **I** and **II**, **5xa** and **5xb**, **7xa** and **7xb**, **7ya** and **7yb**). Similarly, 4-aza-PT derivative endowed with the myristicin moiety afforded higher antimitotic activity than the corresponding 3,5-dimethoxybenzene analog (compare **5xb** and **5xe**). Interestingly, the trend was opposite for the respective 4-oxa-PTs **7xb** and **7xe**. The apiol-derived substituent at C_1 afforded less active compound **7xd**. Introduction of ethylenedioxy fragment to the ring E decreased the antimitotic activity (compare: **5xb** and **5xc**, **7xb** and **7xc**).



Scheme 1. Reagents and conditions: (a) (i) step 1: powdered KOH, $(n\text{-Bu})_4N^+Br^-$, heat, 100 °C, 40 min; (ii) step 2: O_3, $CHCl_3$–MeOH–pyridine (80:20:3 v/v), −15 °C, 1–2 h;[26] (b) C_6H_6–AcOH, reflux, 8 h; (c) CF_3COOH, rt, 24 h; (d) AcOH–$NaBH_3CN$, rt, 15 h; (e) CH_2Cl_2–AcOH–AcOK, 80–85 °C, 5 h;[35] (f) toluene–N_2, Na_2SO_4, reflux, 12–15 h; (g) (i) AcOH–water (1:1 v/v), reflux, 30–40 min; (ii) AcOH–H_2SO_4, 110 °C, 0.5 h. Reactions (a)–(d) were performed as reported previously.[18]

Importantly, it was found that the methylenedioxy ring A could be replaced by methoxy groups to result in retention of activity in both 4-aza- and 4-oxa-series. The position of a methoxy substituent in the ring B played an important role: 6-methoxy derivatives **5xb**, **7xa**, and **7xb** were consistently more potent antimitotic agents than the corresponding 7-methoxy analogs **5yb**, **7ya**, and **7yb**. Three methoxy groups in the B ring (**7zf**) resulted in a dramatic decrease of antiproliferative activity.

Data obtained from the sea urchin embryo assay have been further supported by the Jurkat leukemia cells cytotoxicity test (for experimental details see Supplementary data). Compounds **5xb** and **7xb** were found to be cytotoxic exhibiting IC_{50} values of 18 nM and 55 nM, respectively, versus 25 nM for the parent PT. These molecules, as well as **II**, **5xe**, **5yb**, **7xa**, and **7xc**, were further selected for the NCI60 anticancer drug screen (Tables S1 and S2, Supplementary data). Compounds **7xb** and **7xc** containing the methylenedioxy and ethylenedioxy ring E, respectively, showed similar cytotoxicity. Noticeably, **5xe** was the most potent cell growth inhibitor with GI_{50} <10 nM against 43 cancer cell lines.

When comparing the respective 4-oxa- and 4-aza-PTs **7xb** and **5xb**, it was found that both molecules displayed strong cytotoxicity against a diverse set of cancer cell lines with GI_{50} values in the nanomolar concentration range (Table S1, Supplementary data; PT (NSC 24818) used as a standard). Dose–response profiling further revealed that the aza-derivative **5xb** was more potent than the corresponding oxa-derivative **7xb**. Moreover, **5xb** was more active than the parent PT against a panel of 25 cancer cell lines. Non-small cell lung carcinoma (NCI-H522) and melanoma (MDA-MB-435) were the most sensitive cell lines towards both **5xb** and **7xb** (GI_{50} <10 nM). In addition, **5xb** caused total cell growth inhibition (TGI) of colon (COLO 205) and ovarian cancer (OVCAR-3) cells at concentrations of 26.8 nM and 12.2 nM, respectively. TGI values for **7xb** were 69.2 nM (COLO 205) and 44.6 nM (OVCAR-3). Importantly, both 4-oxa- and 4-aza-derivatives demonstrated high activ-

Table 1
Effects of 4-oxa- and 4-aza-podophyllotoxins on the sea urchin embryos

Compound	EC[a] (nM)		
	Cleavage alteration	Cleavage arrest	Embryo spinning
PT	20	50	200
I[b]	50	500	2000
II[b]	5	50	50
5xa	50	500	500
7xa	5	50	200
5xb[b]	0.5	5	50
7xb	2	10	100
5xc	10	50	1000
7xc	10	50	500
7xd	100	500	4000
5xe[b]	2	10	200
7xe	1	5	50
7ya	100	2000	5000
5yb[b]	10	50	500
7yb	50	200	2000
7zf	2000	>4000	>4000

[a] The sea urchin embryo assay was conducted as described in Ref. 29. Fertilized eggs and hatched blastulae were exposed to twofold decreasing concentrations of compounds. Duplicate measurements showed no differences in effective threshold concentration (EC) values. Experimental details are presented in Supplementary data.
[b] Data from Ref. 18.

Table 2
Growth inhibition of OVCAR-8 and NCI/ADR-RES cell lines

Compound	Cell growth inhibition[a] (GI_{50}, nM)	
	OVCAR-8[b]	NCI/ADR-RES[c]
II	232.0	103.0
7xa	788.0	334.0
5xb	<10	<10
7xb	55.6	18.6
7xc	49.5	23.5
5xe	20.4	<10
5yb	629.0	595.0

[a] GI_{50}: Concentration required for 50% cell growth inhibition.
[b] OVCAR-8: Ovarian cancer cell line 8.
[c] NCI/ADR-RES: P-glycoprotein-overexpressing multi-drug resistant cell line derived from OVCAR-8.[34]

ity against colon cancer cells (Figure S1, Supplementary data). As shown in Table 2, all tested 4-oxa- and 4-aza-PTs were more cytotoxic against NCI/ADR-RES cells overexpresing P-glycoprotein than against the parent OVCAR-8 cell line suggesting a potential for these agents to overcome multi-drug resistance.

In summary, a series of novel 4-oxa-PT derivatives displayed significant microtubule destabilizing activity in the sea urchin embryo assay with EC values in the 10–100 nM range (vs 20 nM for PT). Notable structural features of these active compounds included myristicin-derived or 3′,5′-dimethoxy substitution pattern in the ring E and a 6-methoxy moiety in the ring B, replacing the methylenedioxy ring A. Several 4-oxa-PT derivatives and their aza-analogs (**7xb**, **7xe**, **7yb**, **5xa**, **5xb**, **5xc**) displayed pronounced inhibition of human cancer cell growth in the NCI60 cytotoxicity screen. Moreover, these agents were determined to be active against multi-drug resistant NCI/ADR-RES cells. Although generally 4-aza-PTs were more potent than the respective 4-oxa- derivatives, stability of the latter series towards oxidation may prove to be of importance for the development of anticancer agents with in vivo activity.

Acknowledgments

This work was supported by a Grant from Chemical Block Ltd. We thank the National Cancer Institute (NCI) (Bethesda, MD, USA) for screening compounds **II**, **5xb**, **5xe**, **5yb**, **7xa**, **7xb**, and **7xc** by the Developmental Therapeutics Program at NCI (Anti-cancer Screening Program; http://dtp.cancer.gov).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.01.128.

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