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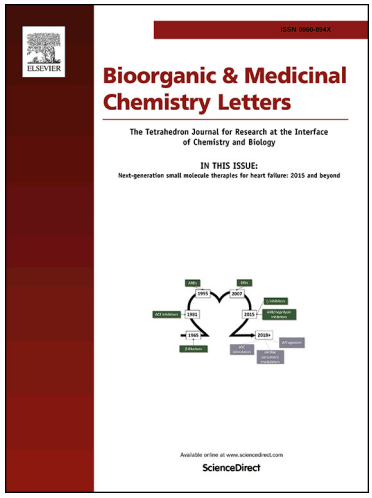

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Graphical Abstract

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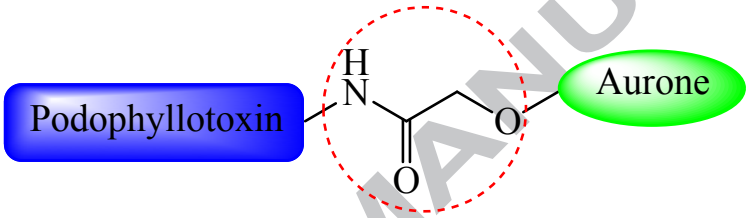

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ABSTRACT

A new series of amide derivatives of 4β-Acetamidobenzofuranone-podophyllotoxin hybrids (**14a-g**) were synthesized and their chemical structures were confirmed by ^{1}H, ^{13}C NMR and mass spectral data. Further, all the synthesized Acetamidobenzofuranone-podophyllotoxin hybrids were evaluated for *in vitro* cytotoxic activity against a panel of four human cancer cell lines i.e., human breast (MCF-7, MDA MB-231), lung (A549), and prostrate (DU-145). Among benzofuranone-podophyllotoxin hybrid compounds, **14b** and **14e** were exhibited more potent activity than standard drug and **14c** and **14f** were showed anticancer activity equivalent to etoposide.

Cancer is a very harmful disease and leading cause of death all over the world.[1] It occurred by the abnormal cell division without manage and are capable to occupy other tissues.[2] There are three major types of treatments are available in the field such as radiation, surgery and chemotherapy. Among them, chemotherapy is the most efficient treatment to devastate the cancer cells without any damaging upshot on the normal cells for various cancers, in which several types of chemotherapeutic agents are used.[3] The human DNA topoisomerse inhibitors are frequently used chemotherapeutic agents.

Podophyllotoxin (**1**) is an antitumor lignan mainly found in the plants *Podophyllum peltatum* and *P. emodi*.[4] It inhibits the microtubule assembly through binding to tubulin (Fig. 1).[5] The biological activity of podophyllotoxin has led to extensive structure modifications resulting in several clinically useful compounds such as etoposide (**2**) and teniposide (**3**) (Fig.1). These are used as DNA topoisomerase II inhibitors in chemotherapy for various types of cancer.[6] However, their acquired drug resistance and poor water solubility hampered their clinical use. To overcome such problems, extensive synthesis efforts have been carried out by research groups to develop NK-611 (**4**)[7] and GL-331 (**5**).[8] Furthermore, these exhibited improved cytotoxicity and topo-II inhibition.[9]

GL-331 induces the apoptotic cell death through independent mechanism and that would also contribute to their cytotoxicity, which was undergone a phase II clinical trials for the treatment of various cancers,[10] and trials are stopped in 2001. In addition, previous reports reveal that GL-331 analogues having electron withdrawing groups on 4β-carbon position is more active.

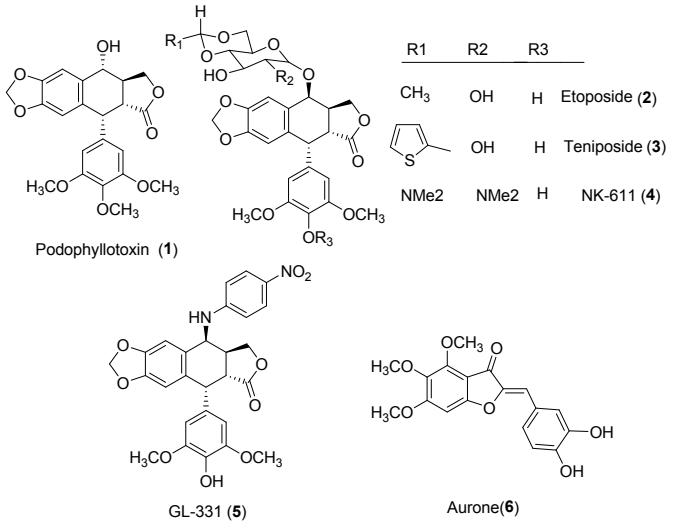


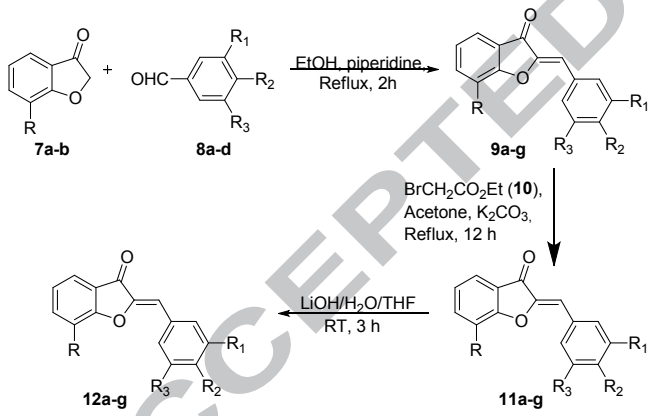
Figure 1. Semi-synthetic derivatives of podophyllotoxin and aurone.

On the other hand, aurone (**6**) is one of most privileged fused bicyclic heterocyclic scaffolds and is isolated from *Uraria*

hamiltonii.[11] Aurones, are secondary metabolite belong to the flavonoids family and are structural isomers of flavones.[12] Several synthesized aurone derivatives are possessed a wide range of biological activities including anticancer,[13,14] tubulin agent,[15] CDK inhibitor,[16] anti-malarial,[17] and acetylcholinesterase inhibitors.[18] Besides, the main reason for the anticancer activity of aurone derivatives was due to the position and the number of hydroxyl groups present on phenyl ring attached to carbon skeleton. In general, aurone derivatives containing the hydroxyl group in *para* position are more potent than that of *ortho* and *meta* positions.

In view of the above impressive biological properties of both podophyllotoxin and aurone scaffolds, we are interested to know the combined effect of both these moieties in a single molecular framework. Hence we would like to synthesize these hybrid molecules to evaluate their anticancer effect.

In furtherance of our research work in the fields of (i) natural product based hybrid molecules,[19-21] NHCs,[22-25] and anti-cancer hybrid molecules,[26-28] herein we report for the first time in this manuscript a facile and new series of aurone-podophyllotoxin hybrids. In the present work, we have synthesized a series of 4*β*-Acetamidobenzofuranone-podophyllotoxin hybrids (**14a-g**) from 4*β*-aminopodophyllotoxin (**13**) and 7-substituted (*Z*)-2-benzylidenebenzofuran-3(2*H*)-one acid linkers (**12a-g**) (Scheme 2). The main chemical differences are the different substituted aurones and substituted aromatic aldehydes with constant two carbons chain acid linker. Further, anti-cancer activity of these derivatives (**14a-g**) were examined towards four human cancer cell lines i.e., human breast (MCF-7, MDA MB-231), lung (A549), and prostrate (DU-145).



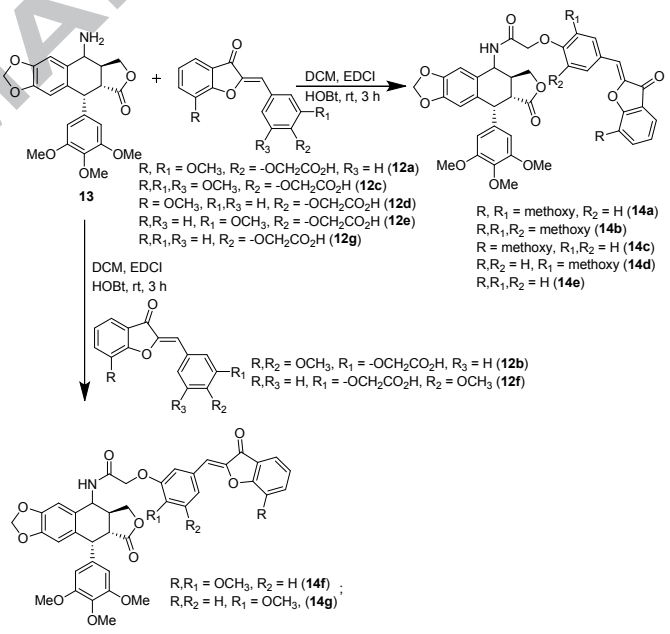
R = H (**7a**), OCH_3, (**7b**)
R_1 = OCH3, R_2 = OH, R_3 = H (**8a**)
R_1 = OH, R_2 = OCH_3, R_3 = H (**8b**)
R_1,R_3 = OCH_3, R_2 = OH (**8c**)
R_1,R_3 = H, R_2 = OH (**8d**)
R, R_1 = OCH_3, R_2 = OH, R_3 = H (**9a**)
R,R_2 = OCH_3, R_1 = OH, R_3 = H (**9b**)
R,R_1,R_3 = OCH_3, R_2 = OH (**9c**)
R = OCH_3, R_1,R_3 = H, R_2 = OH (**9d**)
R,R_3 = H, R_1 = OCH_3, R_2 = OH (**9e**)
R,R_3 = H, R_1 = OH, R_2 = OCH_3 (**9f**)
R,R_1,R_3 = H, R_2 = OH (**9g**)

R, R_1 = OCH_3, R_2 = -OCH_2CO_2Et, R_3 = H (**11a**)
R,R_2 = OCH_3, R_1 = -OCH_2CO_2Et, R_3 = H (**11b**)
R,R_1,R_3 = OCH_3, R_2 = -OCH_2CO_2Et (**11c**)
R = OCH_3, R_1,R_3 = H, R_2 = -OCH_2CO_2Et (**11d**)
R,R_3 = H, R_1 = OCH_3, R_2 = -OCH_2CO_2Et (**11e**)
R,R_3 = H, R_1 = -OCH_2CO_2Et, R_2 = OCH_3 (**11f**)
R,R_1,R_3 = H, R_2 = -OCH_2CO_2Et (**11g**)
R, R_1 = OCH_3, R_2 = -OCH_2CO_2H, R_3 = H (**12a**)
R,R_2 = OCH_3, R_1 = -OCH_2CO_2H, R_3 = H (**12b**)
R,R_1,R_3 = OCH_3, R_2 = -OCH_2CO_2H (**12c**)
R = OCH_3, R_1,R_3 = H, R_2 = -OCH_2CO_2H (**12d**)
R,R_3 = H, R_1 = OCH_3, R_2 = -OCH_2CO_2H (**12e**)
R,R_3 = H, R_1 = -OCH_2CO_2H, R_2 = OCH_3 (**12f**)
R,R_1,R_3 = H, R_2 = -OCH_2CO_2H (**12g**)

Scheme 1. Synthesis of (Z)-2-benzylidenebenzofuran-3(2H)-one acid (**12a-g**).

The synthesis of the (*Z*)-2-benzylidenebenzofuran-3(2*H*)-one acid linker derivatives (**12a-g**) is outlined in Scheme 1. Benzofuran-3(2*H*)-one (**7a**) and 7-methoxybenzofuran-3(2*H*)-one (**7b**) are key intermediates for the preparation of the desired compounds (**9a-g**). The compounds **7a** and **7b** were taken separately and treated with different substituted aromatic aldehydes (**8a-d**) in ethanol and 3 drops of piperidine was added then the mixture was refluxed for 2 hours to afford pure compounds (**9a-g**). The intermediates **9a-g** was reacted with ethyl bromoacetate (**10**) in presence of K_2CO_3 in anhydrous acetone and reaction mixture was stirred at reflux for 12 hours to obtain compounds (**11a-g**). Further, these compounds (**11a-g)** were hydrolyzed under basic condition to afford pure compounds (**12a-g**). Finally, these acid intermediates were subjected to coupling reaction with 4*β*-aminopodophyllotoxin (**13**) in presence of EDCI, HOBt as coupling reagents and stirred at room temperature for 3 hours in anhydrous DCM as a solvent to afford pure corresponding products **14a-g** as shown in Scheme 2.

The synthesized (*Z*)-2-benzylidenebenzofuran-3(2*H*)-one acid linker derivatives (**12a-g**) and 4β-Acetamidobenzofuranone-podophyllotoxin hybrids (**14a-g**) were characterized by $^1H/^{13}C$ NMR and mass spectral analysis (ESI). The absence of ^1H-NMR signals of acid functional group, and emerging of a new signal corresponds to N-H proton of amide provides a good support for the amide coupling to form aurone-podophyllotoxin hybrids. The same features were reflected in ^{13}C-NMR spectra, where the signal belongs to acid carbon was disappeared and a new signal belongs to amide carbon, was appeared after amide coupling.



Scheme 2. Synthesis of 4β-Acetamidobenzofuranone-podophyllotoxin hybrids (**14a-g**).

In vitro cytotoxic activity: All the compounds prepared herein (**14a-g**) were screened for their anti-cancer activity towards four human cancer cell lines including MCF-7 (human breast), A549 (human lung), DU-145 (human prostrate) and MDA MB-231 (human breast) by using MTT assay method and the results acquired were incorporated in Table 1. Etoposide used as standard reference drug and most of tested compound were displayed good to moderate activity with respect to all cell lines. The IC_{50} values of synthesized compounds range from 0.10±0.072 to 8.23±3.61 μM and standard drug showed from 1.91 ± 0.84 to 3.08 ± 0.135 μM. Among them, two compounds, **14b** and **14e** were exhibited excellent activity than etoposide. The other compounds **14c** and **14f** were showed anticancer activity equivalent to etoposide. Further, structure-activity relationship

(SAR) studies of these compounds revealed that the compound **14e** with (R,R_1,R_2 = H) substituent on the furan ring and phenyl ring has showed most promising activity (MCF-7 = 0.13±0.087 µM, A549= 0.10±0.072 µM, DU-145= 0.97±0.068 µM and MDA MB-231= 0.45±0.029 µM) than etoposide. The replacement of (R = H) with (R = methoxy) and R_1,R_2 = methoxy groups resulted compound **14b** has exhibited lower activity on all cell lines (MCF-7 = 0.23±0.081µM, A549= 1.45±0.77µM, DU-145= 1.22±0.69µM and MDA MB-231= 0.87±0.052 µM) when compared with compound **14e**. Where compound **14c** having (R = methoxy, R_1,R_2 = H) groups has displayed good activity on three cell lines (MCF-7 = 1.98±0.87µM, A549= 1.56±0.65µM, DU-145= 2.09±1.59µM and MDA MB-231= 1.68±0.34 µM) when compared with compound **14b**. Whereas, compound **14f** with (R,R_1 = methoxy, R_2 = H) groups has exhibited comparable activity on three cell lines (MCF-7 = 1.87±0.38 µM, DU-145= 2.33±1.76µM and MDA MB-231= 2.18±1.98 µM), respectively. The rest of the compounds **14a, 14d** and **14g** were showed moderate activity on all the cell lines.

MTT assay: Individual wells of a 96-well tissue culture micro titer plate were inoculated with 100 µL of complete medium containing 1×10^4 cells. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 18 hours prior to the experiment. After medium removal, 100 µL of fresh medium containing the test compounds and etoposide at different concentrations such as 0.5, 1, and 2 µM were added to each well and incubated at 37 °C for 24 hours. Then the medium was discarded and replaced with 10 µL MTT dye. Plates were incubated at 37 °C for 2 hours. The resulting formazan crystals were solubilized in 100 µL extraction buffer. The optical density (O.D) was read at 570 nm with micro plate reader (Multi-mode Varioskan Instrument-Themo Scientific). The percentage of DMSO in the medium never exceeded 0.25%.

Table 1
In vitro cytotoxicity of 4β-Acetamidobenzofuranone-podophyllotoxin hybrids (**14a-g**) on human cancer cell lines[a] (IC_{50} µM).[b]

Entry	Compound	MCF-7[c]	A549[d]	DU-145[e]	MDA MB-231[f]
1	14a	2.35±1.60	2.99±2.09	3.98±1.93	2.60±1.75
2	**14b**	**0.23±0.081**	**1.45±0.77**	**1.22±0.69**	**0.87±0.052**
3	14c	1.98±0.87	1.56±0.65	2.09±1.59	1.68±0.34
4	14d	4.51±2.18	2.75±1.85	3.82±2.16	ND
5	**14e**	**0.13±0.087**	**0.10±0.072**	**0.97±0.068**	**0.45±0.029**
6	14f	1.87±0.38	ND	2.33±1.76	2.18±1.98
7	14g	6.23±3.29	7.23±3.41	2.55±0.45	8.23±3.61
8	**Etoposide**	2.11±0.024	3.08±0.135	1.97±0.45	1.91±0.84

"ND" = Not determined.
[a] Each data represents as mean values±SD (standard deviation). [b] From three different experiments performed in triplicates.
[c] MCF-7: human breast cancer cell line. [d] A549: human lung cancer cell line. [e] DU-145: human prostate cancer cell line.
[f] MDA MB-231: human breast cancer cell line.

In conclusion, we have synthesized a new series of amide derivatives of aurone-podophyllotoxin hybrid molecules (**14a-g**) through a facile route and their anticancer activity was demonstrated. These hybrid compounds were tested for their preliminary anticancer activity towards four human cancer cell lines MCF-7 (human breast), A549 (human lung), DU-145 (human prostrate) and MDA MB-231 (human breast) by using MTT assay and etoposide used as standard reference drug. All these hybrid molecules showed good to moderate activity. Among all the synthesized compounds, **14b,** and **14e** were exhibited more potent activity than standard drug. The other compounds **14c** and **14f** were equipotent to etoposide.

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Supplementary data

Supplementary data (experimental procedures and Spectral data of compounds for **9a-g**, **11a-g**, **12a-g** and **14a-g**) associated with this article can be found, in the online version.

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