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Synthesis of 1-*O*-acetylbritannilactone Analogues from *Inula britannica* and *in vitro* Evaluation of their Anticancer Potential

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The natural product 1-*O*-acetylbritannilactone (ABL), a sesquiterpene lactone (STL) isolated from the flowers of the medicinal plant *Inula britannica*, was found to have potent anticancer activity *in vitro*. Previous studies showed that the 6-OH side chain ester analogues of ABL displayed better anticancer activity. In order to look for new natural-product-based anticancer agents, a series of novel ABL analogues was synthesized by N/O-atom installing and aromatic ring esterifying at the 6-OH position of ABL, and their *in vitro* anticancer activities and mechanistic basis of cytotoxicity were presented. Treatment of HeLa cells with representative active compound **4a** showed induction of apoptosis by a biparametric cytofluorimetric analysis and activation of caspase-3. The ability of **4a** to obviously arrest the cell cycle at the G_2/M phase was confirmed by flow cytometric analysis.

Introduction

Natural products are important leads in anticancer drug discovery and always offer a template to derivatize and/or mimic. It has been statistically reported that non-synthetically derived molecules account for 74% of all clinically used anticancer agents since 1940, which almost 50% is either natural products or their direct derivatives.[1,2] Due to natural products often not being optimized for human diseases and often causing unwanted side-effects on many normal cells, biosynthetic and chemical modifications of natural products offer a great opportunity to improve their anticancer properties while reducing their non-specific interactions.[3]

In recent years, the anticancer properties of natural compounds isolated from plants, containing an α-methylene-γ-lactone skeleton, have attracted a lot of attention due to binding thiols of proteins or residues as alkylating reagent.[4,5] Extensive research has been carried out to characterize their molecular mechanisms of action and potential chemotherapeutic application in different types of cancer, such as leukemias, colon cancer. Sesquiterpene lactones (STLs), a group of α-methylene-γ-lactones, are plant-derived compounds, mostly of the Compositae family, used in traditional medicine especially for the treatment of inflammation.[6] However, they exhibit a broad spectrum of other biological effects, including anti-bacterial, anti-helminthic and anti-cancer activity.[5] Besides, STLs are a promising compound class of lead compounds for anticancer drug discovery, such as anti-leukemic parthenolide [7,8] and anti-trypanosomal cynaropicrin (in Fig.1).[9]

1-*O*-acetylbritannilactone (ABL, Fig. 1), a 1,10-*seco*-eudesmanolide sesquiterpene with the α-methylene-γ-lactone skeleton extracted from *Inula britannica* has several biological effects including anticancer activity.[10-16] 1,6-*O*,*O*-diacetylbritannilactone (OABL, Fig. 1) an analogue of ABL, isolated from the same plant, showed better anticancer activity in HL-60 and MCF-7 cells.[10,12,16] Some modified ester analogues by aliphatic side chain esterifying at the 6-OH position of ABL displayed high anticancer activity in our previous report, especially the 12 carbons aliphatic side chain analogue displayed the more potency about 2 to 4 fold improvement toward HCT116, HEp-2 and HeLa cells, compared to OABL.[17] These above results indicated that the 6-OH esterified side chain length is important for the activity of OABL and offers a base for further molecular manipulations.

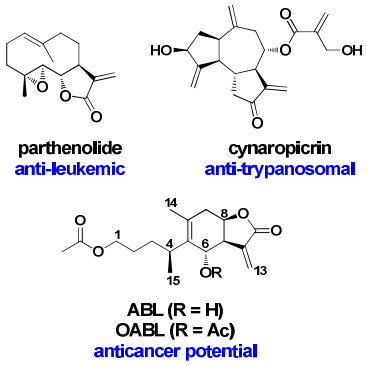


Figure 1. The structures of two sesquiterpene lactones leads (parthenolide and cynaropicrin), 1-*O*-acetylbritannilactone (ABL) and 1,6-*O*,*O*-diacetylbritannilactone (OABL).

1-*O*-acetyl-6-*O*-chloracetylbritannilactone (**2**), with a shorter carbon side chain with a terminal Cl-atom, showed decreased anti-

cancer activity compared to OABL in our previous report.[17] One reason for this compound's decreased activity might be that its side chain is too short and not fit for good binding at the molecular target's packet. Therefore, it is hypothesized by modifying terminal Cl-atom of **2** and the 6-OH of ABL via semi-synthetic approaches to introduce new functionalities in the 6-OH side chain. Recent, many works showed that introduction of hetero-atoms or heterocycle can improve anticancer effects.[18,19] Thus, in the present study, the 6-OH position of ABL was modified with a variety of molecules including further N/O-atom installing and aromatic ring esterifying. Furthermore, their preliminary anticancer potential and mechanistic basis of cytotoxicity were evaluated *in vitro*.

Results and Discussion

Chemistry.

1-*O*-acetylbritannilactone (ABL) and 1,6-*O*,*O*-diacetylbritannilactone (OABL) were isolated from the EtOAc-soluble fraction of the ethanolic extract of the dried flowers of *I. britannica* by repeated column chromatography according to several reported procedure.[10,17] The pure ABL and OABL (white crystals) were obtained by recrystallization from anhydrous EtOH, and the relative configuration of ABL has been revised as 4*S**,6*S** using X-ray diffraction analysis by us. The X-ray diffraction data of OABL was also obtained (Fig. 2) and unambiguously verified the out-of-plane of 4-methyl and the 6α-orientation of the acetyl (OAc) group, which is in accord with the literature data.[20]

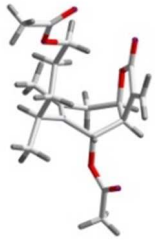
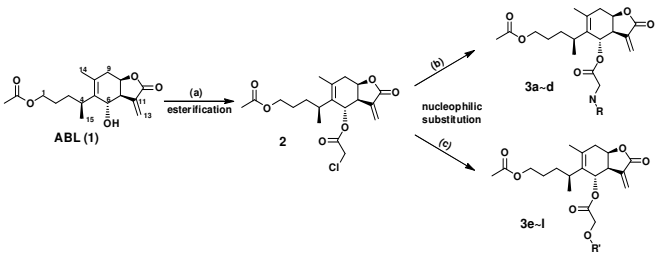


Figure 2. The X-ray derived ORTEP diagram of OABL showed the relative configuration as 4S*,6S*, which is in accord with previous literature.[20]

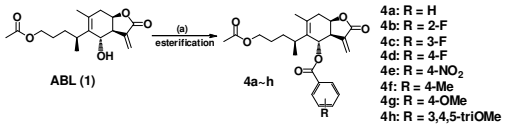
The synthesis of the target compounds **3a–l** was carried out by esterification, followed by nucleophilic substitution. Using ABL as the starting material, the key analogue 1-*O*-acetyl-6-*O*-chloracetylbritannilactone (**2**) was firstly synthesized through a standard esterification procedure. This upon treatment with chloroacetic anhydride in the presence of triethylamine (Et_3N) and catalytic dimethylaminopyridine (DMAP) in dried dichloromethane (DCM) afforded **2** in excellent yield (99%) and in short time (30 min). Nucleophilic substitution was successfully performed by treating **2** with various nucleophiles (containing N/O) for 6–24 h, and yielded the expected compounds **3a–l** in satisfactory yields (Scheme 1). For aromatic esterified anologues **4a–h**, treatment of ABL with various benzoyl chloride in anhydrous pyridine solution gave these target compounds in 80%–99% yields. For a description of chemistry experimental procedures and full characterisation data refer to Supplementary Information.

Biological Activities.

Cytotoxic Activity. *In vitro* anticancer activity of all the semi-synthesized 1-*O*-acetylbritannilactone analogues **3a–l** and **4a–h** were tested *in vitro* against three human cancer cell lines, including HCT116 (human colorectal cancer), HeLa (human cervix cancer) and SGC-7901 (human gastric cancer) using the sulforhodamine B (SRB) assay[21] and the results were compared with ABL, OABL and well-known anticancer drugs etoposide (Vp-16) and 5-fluorouracil (5-Fu). The IC_{50} values (the concentration to cause 50% inhibition of cell viability) of the tested compounds were summarized in Tables 1 and 2.



Scheme 1. Synthetic route of compounds **3a–l**. Reagents and conditions: (a) chloroacetic anhydride, DMAP, Et_3N, CH_2Cl_2, 0 °C to rt, 30 min, 99%; (b) R-NH, CH_3CN, 0 °C to rt, 24 h, 77%–99%; (c) R-OH, K_2CO_3, NaI, TEBA, TEA, acetone, rt, 6–24 h, 33%–99%.



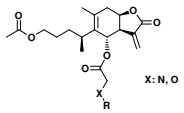
Scheme 2. Synthetic route of compounds **4a–h**. Reagents and conditions: (a) ArCOCl, pyridine, rt, 30 min, 80%–99%.

It can be seen from IC_{50} values of Tables 1 that compounds **3a–d** containing N installing showed slightly better potential than ABL and intermediate **2**, and IC_{50} ranged between 10.3-20.5 μM close to those of OABL (10.1-15.9 μM) against the three human cancer cell lines. Whereas compounds **3e–l** with O atom linking substituted-phenyl exhibited moderate activity, especially for **3h** with strong electron-withdrawing group NO_2 lost the cytotoxic activity (IC_{50} > 100 μM). Compound **3i** with quinolin-8-yloxy group showed similar cytotoxicity with OABL ranging from 8.94 to 14.3 μM on these cancer cells. Compound **3j–k** with different substituted benzo[*d*]isoxazol-6-yloxy group and **3l** bearing 1*H*-benzo[*d*][1,2,3]triazol-6-yl)oxy group, exhibited approximative IC_{50} value for cytotoxicity ranging from 18.3 to 23.7 μM. These data indicated that further chemical modification of **2** by N/O-atom nucleophilic substituent installing may be not a good strategy to improve their anticancer activity.

For aromatic esterified anologues **4a–h**, From Table 2, it can be seen that all compounds generally showed good cytotoxicity. Analogues **4a** and **4f–h** bearing -Me or -OMe group in phenyl showed stronger cytotoxic activity (IC_{50} of 5.19–8.58 μM) compared with that of the parent ABL (32.6 μM) and OABL (11.2 μM) against HeLa cells. Whereas **4b–e** with introduction of electron withdrawing groups (-F or -NO_2) to phenyl had similar activity but had slightly higher IC_{50} than OABL, which imply that introducing electron donating groups in aromatic ring may be useful for improved cytotoxicity. Notably, the **4a** with no substituent group in phenyl showed best activity against HeLa cells with IC_{50} value of 5.19 μM being 2-fold more efficacious than OABL and comparable to Vp-16 and 5-Fu (IC_{50} data of 2.97 μM for Vp-16 and 4.71 μM for 5-Fu, respectively). Whereas 4a respectively showed IC_{50} values of 10.5 and 9.93 μM against HCT116 and SGC-7901 cells with similar IC_{50} of OABL (IC_{50} of 10.1 and 15.9 μM, respectively), suggesting **4a** would exhibit different sensitivity to various cancer cell lines. These

results indicated that aromatic esters may be useful for their anticancer potential improvement against HeLa cells, and **4a** was selected as representative compound for detailed mechanistic investigations in HeLa cells.

Table 1. *In vitro* anticancer activity of 1-*O*-acetylbritannilactone analogues **3a**–**l** in HCT116, HeLa and SGC-7901 cells.



No.	X-R	IC_{50} [a] (μM)		
		HCT116	HeLa	SGC-7901
3a		13.1 ± 0.2	10.7 ± 1.2	11.6 ± 0.1
3b		20.5 ± 0.2	12.7 ± 1.5	18.4 ± 2.1
3c		13.9 ± 0.3	10.3 ± 1.1	11.5 ± 0.8
3d		16.3 ± 2.0	13.9 ± 2.3	14.1 ± 0.9
3e		45.5 ± 3.5	38.6 ± 4.6	46.1 ± 5.3
3f		40.1 ± 3.8	43.9 ± 5.6	49.6 ± 4.6
3g		37.5 ± 2.6	22.9 ± 4.1	27.7 ± 1.6
3h		> 100	> 100	> 100
3i		12.1 ± 1.1	8.94 ± 1.3	14.3 ± 3.1
3j		18.3 ± 1.1	21.7 ± 2.8	21.2 ± 2.3
3k		21.5 ± 2.1	19.2 ± 2.6	19.7 ± 0.4
3l		23.7 ± 3.7	23.0 ± 2.1	21.1 ± 2.9
2	-Cl	18.8 ± 2.3 [b]	28.5 ± 3.1 [b]	26.5 ± 2.9
ABL	-	36.1 ± 3.1 [b]	32.6 ± 2.5 [b]	47.8 ± 5.6
OABL	-	10.1 ± 1.1 [b]	11.2 ± 0.8 [b]	15.9 ± 2.1
5	-	> 100	> 100	> 100
6	-	> 100	> 100	> 100
Vp-16	-	2.13 ± 0.23 [b]	2.97 ± 0.25 [b]	6.56 ± 0.68
5-Fu	-	4.90 ± 0.35	4.71 ± 0.28	0.86 ± 0.05

[a] The IC_{50} values represent the concentration to cause 50% inhibition of cell viability. HCT116, HeLa and SGC-7901 cells were treated with all tested compounds for 72 h. Vp-16 represents etoposide, and 5-Fu represents 5-fluorouracil. All data (mean ± SD) are the average of three or four determinations. [b] These values were cited from ref.[17]

Table 2. *In vitro* anticancer activity of **4a**–**h** in HCT116, HeLa and SGC-7901 cells.

No.	R	IC_{50} [a] (μM)		
		HCT116	HeLa	SGC-7901
4a	H	10.5 ± 2.1	5.19 ± 0.10	9.93 ± 0.06
4b	2-F	21.1 ± 2.4	17.0 ± 0.9	19.3 ± 1.5
4c	3-F	14.5 ± 2.3	11.8 ± 1.1	12.9 ± 3.1
4d	4-F	14.9 ± 2.0	12.0 ± 1.9	13.6 ± 2.5
4e	4-NO_2	ND [b]	11.3 ± 1.8	ND
4f	4-Me	ND	5.46 ± 1.21	ND
4g	4-OMe	ND	8.85 ± 1.92	ND
4h	3,4,5-triOMe	ND	6.54 ± 0.56	ND

[a] The IC_{50} values represent the concentration to cause 50% inhibition of cell viability. HCT116, HeLa and SGC-7901 cells were treated with all tested compounds for 72 h. All data (mean ± SD) are the average of three or four determinations. [b] Not determined.

As mentioned, these compounds possess an α-methylene-γ-lactone motif perhaps having a high tendency to form adducts with thiols of proteins or residues to induce the DNA-fragmentation and apoptosis mediated by glutathione depletion of the cells. In order to see whether such a reaction is responsible for the observed cytotoxicity, we co-administered N-acetyl cysteine (NAC) along with the analogue **4a** during SRB assay. As shown in Fig. 3, NAC (1 mM) completely abolished the concentration-dependent (1.57–12.5 μM) cytotoxicity induced by **4a**, suggesting the possibility of such covalent adduct formation with important cellular targets and α-methylene-γ-lactone motif plays an important role in their anticancer activity.

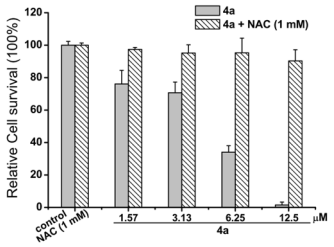


Figure 3. Effect of NAC (1 mM) on cytotoxicity in HeLa cells after exposure to increasing concentrations of **4a** for 72 h. Relative cell survival (100%) was measured by SRB assay. Each experiment was performed in triplicate.

We further characterized the ability of compounds of this class to capture cellular nucleophiles by performing a NMR-based thiol reactivity assay of small molecules described recently.[22] OABL dissolved in DMSO-*d* and the vinyl protons of C-13 were observed by ^{1}H NMR (Fig. 4B). Addition of two molar equivalents of β-mercaptoethanol in the presence of Et_3N resulted in the disappearance of the doublets at δ 5.88 ppm (H_a of OABL) and 6.18 ppm (H_b of OABL) within 3 min, showing high reactivity of the α-methylene-γ-lactone motif by being Michael addition of the β-mercaptoethanol thiol to the α-methylene of OABL and formation of adduct **5** (Fig. 4A). The relative configuration of the newly formed adduct **5** was further confirmed as 11*R** (Fig. 4C) through NOESY spectra (see Supplementary Information). Because of β-configuration for H-6 of OABL, NOESY correlation observed apparently between the H-6 (δ = 5.37 ppm) and H-11 (δ = 2.50 ppm) established the H_{11} β-configuration, staying in tune with the literature precedent [23-25] that the Michael addition at exocyclic double bond of sesquiterpene lactones proceeds with high diastereoselectivity at C-11 position giving preferentially the less hindered *R* isomer. When **4a** and β-mercaptoethanol were conducted in DMSO-*d* in the above condition (Fig. 4A), the similar quick disappearance of the doublets of H-13a and H-13b of **4a** and formation of adduct **6** were observed within 3 min (Fig. S2 in Supplementary Information). However, cytotoxicity assay showed that the adduct **5** and **6** lost the anticancer potential against these cancer cell lines (IC_{50} values > 100 μM in Table 1), also revealing the importance of the α-methylene-γ-lactone motif for their anticancer activity.

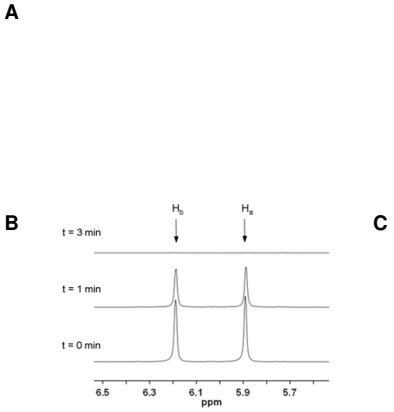


Figure 4. (A) Reaction of OABL or **4a** with β-mercaptoethanol to forms Michael adducts **5** and **6**. (B) ^{1}H NMR analysis of vinyl protons H_a (5.88 ppm) and H_b (6.18 ppm) from OABL before (t = 0 min) and after addition of β-mercaptoethanol and Et_3N in DMSO-*d*6 at 25 °C. Reaction was judged to be nearly complete after 3 min. (C) Selected NOE correlations of adduct **5**. All spectra were taken at 500 MHz.

Apoptosis Assay. Due to the excellent cytotoxicity of **4a** against HeLa cancer cell line, it was chosen to be further investigated regarding its mechanism of action. To test whether the inhibition of cell growth was related to cell apoptosis, we performed a flow cytometric analysis by staining cells with annexin V–FITC and propidium iodide (PI).[26] The result showed that treatment with 10 μM OABL for 72 h induced 6.9% and 24.4% early and late apoptotic cells, respectively (Fig. 5). Similar apoptosis induced by OABL have been reported previously in another human cancer cells.[27,28]After treatment with compound **4a** at two low concentrations (3 and 6 μM), the early and late apoptosis rates were significantly increased from 3.9% and 6.9% (for 3 μM group) to 4.5% and 30.0% (for 6 μM group), respectively. The results indicated that **4a** caused a markedly increased the cellular apoptosis at lower concentration**.**

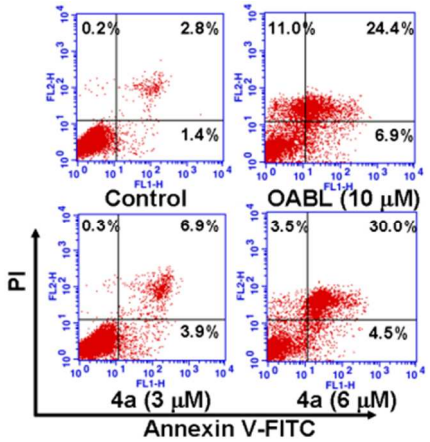


Figure 5. Induction of apoptosis by OABL and compound **4a** at the indicated concentrations on HeLa cells. Flow cytometric analysis of viable, apoptotic, and necrotic cells after 72 h of treatment with OABL and **4a**. Cells in the lower left quadrants are alive, in the lower right quadrants are in early apoptosis, in the upper right quadrants are in late apoptosis, and in the upper left quadrants are necrotic. Percentage of total signal within the quadrant is indicated. Each experiment was performed in triplicate.

Apoptosis induction is characteristic of many known anticancer agents, and apoptosis signals will finally lead to the cleaved processing of procaspase-3 to caspase-3, which assist the degradation of cellular proteins.[29] We investigated the presence of cleaved caspase-3 after treating HeLa cells with **4a** at different concentrations (3, 6, 12 μM). The western blot result showed that presence of cleaved caspase-3 after incubating HeLa cells with Vp-16 (3 μM) and **4a** in a concentration-dependent manner (Fig. 6). Presence of this enzyme in cells treated with **4a** clearly indicated the processing of procaspase-3 and induction of apoptosis.

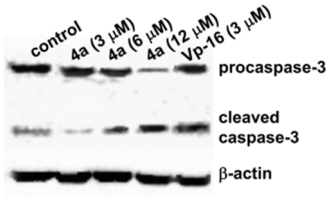


Figure 6. Changes of caspase-3 in the induction of apoptosis triggered by **4a** on HeLa cells. HeLa cells treated with **4a** and positive control Vp-16 at the indicated concentration for 48 h were lysed. The lysate was resolved on a 10% SDS-PAGE, transferred on to a nitrocellulose membrane and probed for cleaved caspase-3. β-actin was used as a loading control.

Cell Cycle Analysis. To determine whether the high inhibitory effect of **4a** was caused by cell cycle accumulation at a certain phase, we also evaluated effect of **4a** on the cell cycle distribution of HeLa cells with propidium iodide (PI) by flow cytometry.[30] As shown in Fig. 7, treatment with **4a** for 24 h at different concentrations (5, 10, 20 μM), the percentage of cells in G2/M phase at different concentrations were 29.2%, 53.2% and 63.7%, respectively, as compared to 28.4% in control cells, suggesting that **4a** caused an obvious G2/M arrest with a concomitant decrease in terms of the number of cells in the G0/G1 and S phases. This result is similar to previous reports of OABL-induced cell cycle arrest in G2/M phase on colorectal cancer cells and breast cancer cells,[12,17] revealing that the superior cytotoxicity of **4a** over OABL and **2** was associated with a mechanism similar with that of OABL in cell cycle progression.

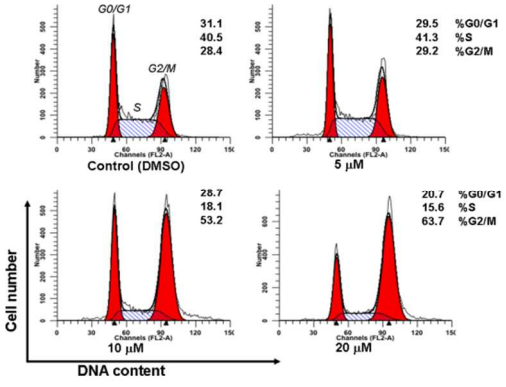


Figure 7. Effect of compound **4a** on cell cycle distribution in HeLa cells. Cells were treated with the indicated concentrations for 24 h, then harvested, and analyzed by flow cytometry.

Conclusions

In summary, we have described the synthesis of a series of new ABL analogues through further N/O-atom installing and aromatic ring

esterifying at the 6-OH position, and evaluated their *in vitro* anticancer effects against three human cancer cell lines (HCT116, HeLa and SGC-7901). The present study indicated that introduction of phenyl group at 6-OH of ABL and retention of the α-methylene-γ-lactone motif lead to an increase in the activity. The representative analogue **4a** induced apoptosis characterized by a biparametric cytofluorimetric analysis and activation of caspase-3 against HeLa cells. Subsequent flow cytometric analysis involving this compound clearly showed that they are capable of arresting the cell cycle at G2/M phase on HeLa cells. The results indicated that **4a** could act as an anticancer potential hit against HeLa cells and would be further worth being developed into new anticancer leads by proper structure optimization.

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Notes and references

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Electronic Supplementary Information (ESI) available: [all experimental procedures and NMR, ESI-MS, X-Ray and HPLC purity data and selected 1D/2D NMR spectra]. See DOI: 10.1039/c000000x/

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Synthesis of 1-*O*-acetylbritannilactone Analogues from *Inula britannica* and *in vitro* Evaluation of their Anticancer Potential

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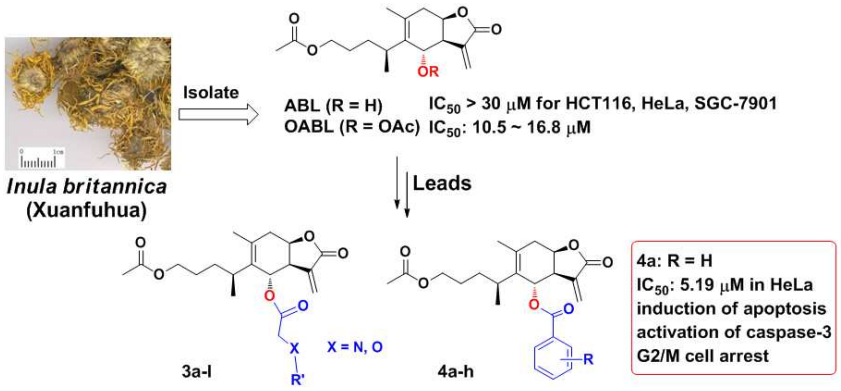
Graphical abstract



A series of novel ABL analogues was synthesized by N/O-atom installing and aromatic ring esterifying, and **4a** showed *in vitro* markedly anticancer activities against HeLa cells associated with induction of apoptosis, activation of caspase-3 and G2/M cell arrest.